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(57) Abstract

Novel fusion polypeptide ligands that bind Eph family receptors or the Tie-2 receptor are identified, and methods for making the fusion polypeptide ligands in biologically active form are described. Nucleic acids encoding these novel fusion polypeptide ligands enable production of the fusion polypeptide ligands. The method of making the nucleic acids and the fusion polypeptide ligands is broadly applicable to the production of polypeptide ligands in general, resulting in improved affinity and/or increased activity of the ligand when compared to its native form.

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METHOD OF ENHANCING THE BIOLOGICAL ACTIVITY OF LIGANDS

This application claims priority of U.S. Application No. 60/113,387, filed December 23, 1998. Throughout this application, various publications are cited. The disclosures of each and all of those publications are hereby incorporated by reference in their entireties into this application.

INTRODUCTION

The present invention provides for novel methods for producing novel fusion polypeptide ligands that have enhanced biological activity as compared to the polypeptide ligands in their native form. The invention also provides for nucleic acids useful for producing biologically active fusion polypeptide ligands, and the fusion polypeptide ligands themselves.

BACKGROUND OF THE INVENTION

The ability of polypeptide ligands to bind cells and thereby elicit a phenotypic response such as cell growth, survival or differentiation is often mediated through transmembrane tyrosine kinases. The extracellular portion of each receptor tyrosine kinase (RTK) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic. Binding of a ligand to the extracellular domain results in signal transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates (Mohammadi, et al.,1990, Mol. Cell. Biol., 11: 5068-5078; Fantl, et al., 1992, Cell, 69:413-413).

RTKs appear to undergo dimerization or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci.

13:443-447; Ullrich and Schlessinger, 1990, Cell, 61:203-212; Schlessinger and Ullrich, 1992, Neuron 9:383-391); molecular interactions between dimerizing cytoplasmic domains lead to activation of kinase function. In some instances, such as the growth factor platelet derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al. , 1988, Science, 240: 1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of EGF, the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem., 259:14631-14636).

Throughout the history of the biotechnology industry, many novel genes and associated proteins have been identified by virtue of their sequence homology with known genes. Many such proteins are purported to be receptors, but since their cognate ligands have not been identified, they are referred to as orphan receptors. The screening of many of these orphan receptors often leads to the identification of ligands that are capable of binding to the receptor, although the binding is often not associated with activation of any intracellular kinases or any other phenotypic change. Such was the case for members of the Eph receptor family. For sake of clarity, applicants incorporate by reference herein a letter cited as Eph Nomenclature Committee, 1997, published in Cell vol. 90: 403-403 (1997) which sets forth a nomenclature for the Eph Receptor and Eph Ligand Families.

Little, if any, biological activity had been observed in response to binding of a ligand to an Eph family receptor prior to the discovery as set forth in U.S. Patent No. 5,747,033 issued May 5, 1998. That patent describes the concept of "clustering" whereby the soluble domains of ligands were combined to create multimers capable of activating the cognate receptors. Applicants have now extended the concept of clustering to additional ligands outside the Eph family, for example, the Tie-2 receptor ligands known as the angiopoietins, and have also discovered that this method for production of homogeneous forms of clustered ligands is broadly applicable to improve

the affinity and/or increase the activity of a ligand as compared to the native form of the ligand.

Angiopoietin-1 (Ang) is one of two known ligands for the Tie-2 receptor and has been shown to be an agonist for Tie-2 (Davis, et al, 1996, Cell 87:1161-1169), whereas the second known ligand, angiopoietin-2, has been shown to be a naturally occurring antagonist of the Tie-2 receptor (Maisonpierre, et al., 1997, Science 277:55-60). Ang1* is a mutant form of angiopoietin-1 that comprises the N-terminal domain of angiopoietin-2 fused to the coiled-coil domain and the fibrinogen domain of angiopoietin-1 and that has a Cys to Ser mutation at amino acid 245. Ang1* has been shown to be a potent agonist for the Tie-2 receptor.

Experiments with mutants of angiopoietin-1 and angiopoietin-2 have demonstrated that the fibrinogen domains (FD) are the receptor-binding domains, and that dimerized versions of, for example Ang-1-FD-Fc, (i.e., the fibrinogen domain of Ang-1 fused to an Fc domain), can bind to the Tie-2 receptor with much higher affinity than monomeric Ang-1-FD (dimerization occurs due to the interaction between the Fc components of adjacent molecules). However, Ang-1-FD-Fc is not able to induce phosphorylation (activate) the Tie-2 receptor on endothelial cells unless it is further clustered with goat anti-human Fc antibodies (Jackson Immunoresearch). For this reason, mutant versions of Ang-1-FD and Ang-2-FD (i.e., the fibrinogen domain of Ang-2) were designed that were intrinsically more highly clustered.

SUMMARY OF THE INVENTION

The present invention provides for novel, biologically active, soluble forms of polypeptide ligands that bind to receptors on cells. Such polypeptide ligands are useful in promoting a differential function and/or influencing the phenotype, such as growth and/or proliferation, of receptor-bearing

cells. The invention also provides for nucleic acids encoding such polypeptide ligands, and both prokaryotic and eukaryotic expression systems for producing such polypeptide ligands. According to the invention, soluble forms of the polypeptide ligands described herein may be used to promote biological responses in receptor-expressing cells. In particular, a general method is described herein which produces fusion polypeptide ligands that may then be clustered, which functions to make otherwise inactive soluble polypeptide ligands biologically active, or which enhances the biological activity of polypeptide ligands that, absent such clustering, would have lower levels of biological activity. This method may be used to cluster a plurality of (more than one) receptor binding domains from any ligand which has improved affinity and/or increased activity (i.e. signaling ability) when clustered as compared to the native form of the ligand.

DESCRIPTION OF THE FIGURES

Figure 1A-1E - Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-FD-Fc.

Figure 2A-2E - Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-FD-Fc.

Figure 3A-3E - Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-Fc-FD.

Figure 4A-4E - Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-Fc-FD.

Figure 5 - Molecular Weight Analysis of Ang-1-FD-Fc-FD protein. SDS PAGE analyses showing a band running at about 210kD under non-reducing conditions (lane 3) and a band running at about 85kD under reducing conditions (lane 7).

Figure 6 - Light scatter analysis to confirm the molecular weight of Ang-1-FD-Fc-FD and to determine whether or not the protein is a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent is monitored with an on line light scattering detector and a refractive index and/or a UV detector. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) is used to calculate the protein concentration. The molecular weight of protein is then calculated from the angular dependence of light scattering. The molecular weight of the dimeric protein appears to be approximately 200kD and presence of a single peak implies that the protein solution is homogenous.

Figure 7 - Molecular Weight Analysis of Ang-2-FD-Fc-FD. SDS PAGE analyses showing a band running at about 200kD under non-reducing conditions (lanes 7 and 8) and a band running at about 88kD under reducing conditions (lanes 3 and 4).

Figure 8 - Light scatter analysis to confirm the molecular weight of Ang-2-FD-Fc-FD and to determine whether or not the protein is a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent is monitored with an on line light scattering detector and a refractive index and/or a UV detector. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) is used to calculate the protein concentration. The molecular weight of protein is then calculated from the angular dependence of light scattering. The molecular weight of the dimeric protein appears to be approximately 171kD and presence of a single peak implies that the

protein solution is homogenous.

Figure 9 - Ang1*-mediated vs. Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. A standard phosphorylation assay revealed that Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926.

Figure 10 - Ability of Ang-2-FD-Fc-FD to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells. In a standard phosphorylation assay, Ang-2-FD-Fc-FD is able to block Ang1* stimulation of the Tie-2 receptor when it is present in at least a 10-15 fold molar excess of Ang1*.

Figure 11 - Ability of angiopoietin-2 to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells. In a standard phosphorylation assay, at a 20 fold molar excess, angiopoietin-2 is not able to reduce the Ang1*-mediated phosphorylation level to 50%. This result, coupled with the results described in Figure 10 implies that Ang-2-FD-Fc-FD is a more potent inhibitor of Ang1*-mediated Tie-2 receptor phosphorylation than angiopoietin-2.

Figure 12 - Ability of Ang-2-FD-Fc-FD to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells. In a standard phosphorylation assay, it is shown that while there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, Ang-2-FD-Fc-FD seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 10.

Figure 13 - Ability of angiopoietin-2 to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells. In a standard phosphorylation assay, it is shown that there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, but, like Ang-2-FD-Fc-FD, angiopoietin-2 seems to be more effective at

blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 11.

Figure 14A-14E - Nucleic acid sequence and deduced amino acid sequence of Ephrin-B1-Ephrin-B1-Fc.

Figure 15A-15E - Nucleic acid sequence and deduced amino acid sequence of Ephrin-B2-Ephrin-B2-Fc.

Figure 16 - Comparison of Ephrin-B1-Fc, Ephrin-B1-Ephrin-B1-Fc, Ephrin-B2-Fc and Ephrin-B2-Ephrin-B2-Fc in standard EphB2 phosphorylation assays. COS cells were serum-starved and then left untreated (UT), lane 1, or were treated with unclustered and clustered Ephrin-B1-Fc (Efn-B1), lanes 2 and 3. COS cells were also treated with unclustered and clustered Ephrin-B1-Ephrin-B1-Fc (Efn-B1 DD), lanes 4 and 5. In addition cells were likewise treated with unclustered and clustered Ephrin-B2-Fc (Efn-B2), lanes 6 and 7 and with unclustered and clustered Ephrin-B2-Ephrin-B2-Fc (Efn-B2 DD), lanes 8 and 9. The extent of EphB2 phosphorylation was assessed by anti-phosphotyrosine western blotting (upper panels) and the relative amounts of EphB2 in each lane was determined by anti-EphB2 western blotting (lower panels).

Figure 17 - Ang1*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were stimulated with 0.4 μg/ml Ang1* or 0.2 μg/ml or 0.4 μg/ml stable CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells.

Figure 18 - Ability of stable CHO clone-derived Ang-2-FD-Fc-FD to block stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were treated with 0.2

μg/ml of the Tie-2 agonist Ang-1-FD-Fc-FD and 2 μg/ml, 4 μg/ml, 8 μg/ml or 16 μg/ml of stable CHO clone-derived Ang-2-FD-Fc-FD. Ang-2-FD-Fc-FD is able to block or stable CHO clone-derived Ang-1-FD-Fc-FD stimulation of the Tie-2 receptor when it is present in at least a 40 fold molar excess of stable CHO clone-derived Ang-1-FD-Fc-FD.

DETAILED DESCRIPTION OF THE INVENTION

As described in greater detail below, applicants have discovered a method for "clustering" polypeptide ligands, which functions to make otherwise inactive soluble polypeptide ligands biologically active, or which enhances the biological activity of polypeptide ligands that, absent such clustering, would have lower levels of biological activity. This method may be used to cluster a plurality of (more than one) receptor binding domains from any ligand which has improved affinity and/or increased activity (i.e. signaling ability) when clustered as compared to the native form of the ligand.

The present invention provides for a nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of the receptor binding domain of a ligand, the first subunit being fused to the N-terminal end of a multimerizing component, said multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of a ligand.

In one embodiment of the invention, the receptor binding domains of the first and second subunits are copies of the receptor binding domain of the same ligand. The first and second subunits may each have one or more than one copy of the receptor binding domain of the ligand. In specific embodiments of the invention, the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2. Alternatively, the

receptor binding domain is from a ligand selected from the group consisting of the EPH family of ligands (i.e., the ephrins).

In another embodiment of the invention, the receptor binding domains of the first subunit are copies of the receptor binding domain of a different ligand from the receptor binding domains of the second subunit. For example, the first subunit may comprise the receptor binding domain of an angiopoietin and the second subunit may comprise the receptor binding domain of vascular endothelial growth factor (VEGF). Alternatively, the first subunit may comprise the receptor binding domain of VEGF and the second subunit may comprise the receptor binding domain an angiopoietin. Still further, the first and second subunits may each have one or more than one copy of the receptor binding domain of their respective ligand.

By "receptor binding domain" what is meant is the minimal portion of the ligand that is necessary to bind its receptor.

In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(ΔC1).

The present invention also provides for fusion polypeptides encoded by the nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are described in European Patent Application of ZymoGenetics, Inc., Publication No. EP 0 721 983 A1 published 17 July 1996

and include S. cerevisiae repressible acid phosphatase (Mizunaga et al., 1988, J. Biochem. (Tokyo) 103:321-326); the S. cerevisiae type 1 killer preprotoxin (Sturley et al., 1986, EMBO J. 5:3381-3390); the S. calsbergensis alpha galactosidase melibiase (Sumner-Smith, et al., 1985, Gene 36:333-340); and the Neurospora crassa ornithine decarboxylase (Digangi, et al., 1987, J. Biol. Chem. 262:7889-7893). Sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); the S. cerevisiae SUC2 gene (Carlson et al., 1983, Mol. Cell. Biol. 3:439-447); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS or CHO cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The fusion polypeptides useful for practicing the present invention may be prepared by expression in a prokaryotic or eukaryotic expression system.

The recombinant gene may be expressed and the polypeptide purified utilizing any number of methods. The gene may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The fusion polypeptides may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The present invention also provides for a nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises more than one copy of the receptor binding domain of a ligand in tandem, and wherein either the N-terminal or the C-terminal receptor binding domain is also fused to a multimerizing component. In one embodiment of the invention, the receptor binding domains are fused contiguously. In another embodiment of the invention, the receptor binding domains are from a ligand that is not a member of the EPH family of ligands (i.e., not an ephrin). In specific embodiments of the invention, the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2. In an alternative embodiment, the receptor binding domain is from vascular endothelial growth factor (VEGF). In another embodiment, the receptor binding domain is from an ephrin.

By "receptor binding domain" what is meant is the minimal portion of the ligand that is necessary to bind its receptor.

In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(ΔC1).

The present invention also provides for fusion polypeptides encoded by the nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are described in European Patent Application of ZymoGenetics, Inc., Publication No. EP 0 721 983 A1 published 17 July 1996 and include S. cerevisiae repressible acid phosphatase (Mizunaga et al., 1988, J. Biochem. (Tokyo) 103:321-326); the S. cerevisiae type 1 killer preprotoxin (Sturley et al., 1986, EMBO J. 5:3381-3390); the S. calsbergensis alpha galactosidase melibiase (Sumner-Smith, et al., 1985, Gene 36:333-340); and the Neurospora crassa ornithine decarboxylase (Digangi, et al., 1987, J. Biol. Chem. 262:7889-7893). Sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); the S. cerevisiae SUC2 gene (Carlson et al., 1983, Mol. Cell. Biol. 3:439-447); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is

operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS or CHO cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The fusion polypeptides useful for practicing the present invention may be prepared by expression in a prokaryotic or eukaryotic expression system. The recombinant gene may be expressed and the polypeptide purified utilizing any number of methods. The gene may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The fusion polypeptides may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The Examples describe the preparation of novel polypeptide ligands that comprise a receptor binding domain of a member of the Eph (Eph transmembrane tyrosine kinase family ligands) family of ligands or of a

member of the angiopoietin family of ligands that can bind the Tie-2 receptor.

For a description of novel Eph family ligands, methods of making and using them, as well as the sequences of EHK-1L, B61 and ELK-L, together with a description of a method of enhancing the biological activity of EPH family ligands by clustering them, applicants refer to U.S. Patent No. 5,747,033 issued on May 5, 1998 which is hereby incorporated by reference in its entirety. Applicants further refer to International Application PCT/US93/10879, published as WO 94/11020 on May 26, 1994; and International Application PCT/US96/17201 published as WO 97/15667 entitled "Biologically Active EPH Family Ligands" each of which is hereby incorporated by reference in its entirety.

As has been previously reported, a family of ligands for the TIE-2 receptor has been discovered and named the Angiopoietins. This family, consisting of TIE-2 ligand 1 (Ang-1); TIE-2 ligand 2 (Ang-2); TIE ligand 3 (Ang-3); and TIE ligand 4 (Ang-4) has been extensively characterized. For a description of the cloning, sequencing and characterization of the angiopoietins, as well as for methods of making and uses thereof, including the production and characterization of modified and chimeric ligands thereof, reference is hereby made to the following publications, each of which is incorporated by reference herein in its entirety: U.S. Patent No. 5,521,073 issued May 28, 1996; U.S. Patent No. 5,643,755 issued July 1, 1997; U.S. Patent No. 5,650,490 issued July 22, 1997; U.S. Patent No. 5,814,464 issued September 29, 1998; U.S. Patent No. 5,879,672 issued March 9, 1999; U.S. Patent No. 5,851,797 issued December 22, 1998; PCT International Application entitled "TIE-2 Ligands Methods of Making and Uses Thereof," published as WO 96/11269 on 18 April 1996 in the name of Regeneron Pharmaceuticals, Inc.; PCT International Application entitled "TIE-2 Ligands Methods of Making and Uses Thereof,"published as WO 96/31598 on 10 October 1996 in the name of Regeneron Pharmaceuticals, Inc.; PCT International Application entitled

"TIE-2 Receptor Ligands (TIE Ligand-3; TIE Ligand-4) And Their Uses," published as WO 97/48804 on 24 December 1997 in the name of Regeneron Pharmaceuticals, Inc; and PCT International Application entitled "Modified TIE-2 Receptor Ligands," published as WO 98/05779 on 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc.

When used herein, fusion polypeptide includes functionally equivalent molecules in which amino acid residues are substituted for residues within the sequence resulting in a silent or conservative change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent or conservative alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are proteins or fragments or derivatives thereof which exhibit the same or similar biological activity and derivatives which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Cells that express the fusion polypeptides of the invention are genetically engineered to produce them by, for example, transfection, transduction, electroporation, or microinjection.

The present invention encompasses the nucleic acid sequences encoding the fusion polypeptides of the invention, as well as sequences that hybridize under stringent conditions to nucleic acid sequences that are complementary to the nucleic acid sequences of the invention. Stringent conditions are set forth in, for example, Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2 ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989). In addition, the present invention encompasses nucleic acid sequences that are different from the nucleic acid sequences of the invention but which nevertheless encode the fusion polypeptides of the invention due to the degeneracy of the genetic code.

In addition, the present invention contemplates use of the fusion polypeptides described herein in tagged forms.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the fusion polypeptides of the invention using appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence encoding the fusion polypeptides of the invention may be regulated by a second nucleic acid sequence so that the fusion polypeptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the fusion polypeptides described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the fusion polypeptide include, but are not limited to the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980,

Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene

control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising Eph fusion polypeptide encoding or angiopoietin fusion polypeptide encoding nucleic acids as described herein, are used to transfect the host and thereby direct expression of such nucleic acid to produce fusion polypeptides which may then be recovered in biologically active form. As used herein, a biologically active form includes a form capable of binding to the relevant receptor and causing a differentiated function and/or influencing the phenotype of the cell expressing the receptor. Such biologically active forms would, for example, induce phosphorylation of the tyrosine kinase domain of the Ehk-1, Elk, or Tie2 receptor, or stimulation of synthesis of cellular DNA.

Expression vectors containing the nucleic acid inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign nucleic acids inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted nucleic acid sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign nucleic acid sequences in the vector. For example, if an efl nucleic acid sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign nucleic acid product expressed by the recombinant. Such assays can be based, for example, on the physical or

functional properties of the nucleic acid product of interest, for example, by binding of a ligand to a receptor or portion thereof which may be tagged with, for example, a detectable antibody or portion thereof or binding to antibodies produced against the protein of interest or a portion thereof.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the ephrin or angiopoietin fusion polypeptide as described herein.

The ephrin fusion polypeptides of the invention may be useful in methods of treating a patient suffering from a neurological disorder comprising treating the patient with an effective amount of the ephrin fusion polypeptide.

For example, the Elk receptor is expressed primarily in brain. Accordingly, it is believed that an Elk binding ephrin fusion polypeptide ligand will support the induction of a differential function and/or influence the phenotype, such as growth and/or survival of neural cells that express this receptor.

The present invention also provides for pharmaceutical compositions comprising the ephrin fusion polypeptide in a suitable pharmacologic carrier. The compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

As our understanding of neurodegenerative disease/neurotrauma becomes clearer, it may become apparent that it would be beneficial to decrease the effect of endogenous Efl-6. Therefore, in areas of nervous system trauma, it may be desirable to provide Efl-6 antagonists, including, but not limited to,

fusion polypeptide forms of Efl-6 which may compete with cell-bound ligand for interaction with Elk receptor. It may be desirable to provide such antagonists locally at the injury site rather than systemically. Use of an Efl-6 antagonist providing implant may be desirable.

Alternatively, certain conditions may benefit from an increase in Efl-6 responsiveness. It may therefore be beneficial to increase the number or binding affinity of Efl-6 in patients suffering from such conditions.

The invention herein further provides for the development of a fusion polypeptide, as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the TIE-2 receptor. Such molecules may be used in a method of treatment of the human or animal body, or in a method of diagnosis.

Because TIE-2 receptor has been identified in association with endothelial cells and, as was previously demonstrated, blocking of agonists of the receptor such as TIE-2 ligand 1 (Ang-1) has been shown to prevent vascularization, applicants expect that TIE-2 agonist fusion polypeptides of the invention may be useful for the induction of vascularization in diseases or disorders where such vascularization is indicated. Such diseases or disorders would include wound healing, ischemia and diabetes. The ligands may be tested in animal models and used therapeutically as described for other agents, such as vascular endothelial growth factor (VEGF), another endothelial cell-specific factor that is angiogenic. Ferrara, et al. U.S. Patent No. 5,332,671 issued July 26, 1994. The Ferrara reference, as well as other studies, describe <u>in vitro</u> and <u>in vivo</u> studies that may be used to demonstrate the effect of an angiogenic factor in enhancing blood flow to ischemic myocardium, enhancing wound healing, and in other therapeutic settings wherein neoangiogenesis is desired. [see Sudo, et al., European Patent Application 0 550 296 A2 published July 7, 1993; Banai, et al. Circulation 89:2183-2189 (1994); Unger, et al. Am. J. Physiol. 266:H1588-H1595

(1994); Lazarous, et al. Circulation 91:145-153 (1995)]. According to the invention, the agonist fusion polypeptides may be used alone or in combination with one or more additional pharmaceutically active compounds such as, for example, VEGF or basic fibroblast growth factor (bFGF).

Conversely, antagonists of the TIE-2 receptor, such as TIE-2 receptorbodies or TIE-2 ligand 2 (Ang-2) as described in Example 9 in International Publication No. WO 96/31598 published 10 October 1996, have been shown to prevent or attenuate vascularization, and are thus expected to be useful in preventing or attenuating, for example, tumor growth. Similarly then, TIE-2 antagonist fusion polypeptides of the invention would also be useful for those purposes. These antagonists may be used alone or in combination with other compositions, such as anti-VEGF antibodies, that have been shown to be useful in treating conditions in which the therapeutic intent is to block angiogenesis.

For example, applicants have determined that TIE-2 ligands are expressed in cells within, or closely associated with, tumors. For example, TIE-2 ligand 2 (Ang-2) appears to be tightly associated with tumor endothelial cells. Accordingly, TIE-2 antagonist fusion polypeptides of the invention may also be useful in preventing or attenuating, for example, tumor growth.

In other embodiments, the TIE-2 agonist fusion polypeptides of the invention described herein may be used as hematopoietic factors. A variety of hematopoietic factors and their receptors are involved in the proliferation and/or differentiation and/or migration of the various cells types contained within blood. Because the TIE-2 receptors are expressed in early hematopoietic cells, the TIE-2 ligands are expected to play a comparable role in the proliferation or differentiation or migration of these cells. Thus, for example, TIE-2 agonist fusion polypeptide compositions may be prepared, assayed, examined in in vitro and in vivo biological systems and

used therapeutically as described in any of the following: Sousa, U.S. Patent No. 4,810,643, Lee, et al., Proc. Natl. Acad. Sci. USA 82:4360-4364 (1985) Wong, et al. Science, 228:810-814 (1985); Yokota, et al. Proc. Natl. Acad. Sci (USA) 81:1070 (1984); Bosselman, et al. WO 9105795 published May 2, 1991 entitled "Stem Cell Factor" and Kirkness, et al. WO 95/19985 published July 27, 1995 entitled "Haemopoietic Maturation Factor". Accordingly, the fusion polypeptides may be used to diagnose or treat conditions in which normal hematopoiesis is suppressed, including, but not limited to anemia, thrombocytopenia, leukopenia and granulocytopenia. In a preferred embodiment, the fusion polypeptides may be used to stimulate differentiation of blood cell precursors in situations where a patient has a disease, such as acquired immune deficiency syndrome (AIDS) which has caused a reduction in normal blood cell levels, or in clinical settings in which enhancement of hematopoietic populations is desired, such as in conjunction with bone marrow transplant, or in the treatment of aplasia or myelosuppression caused by radiation, chemical treatment or chemotherapy.

The fusion polypeptides of the present invention may be used alone, or in combination with another pharmaceutically active agents such as, for example, cytokines, neurotrophins, interleukins, etc. In a preferred embodiment, the fusion polypeptides may be used in conjunction with any of a number of factors which are known to induce stem cell or other hematopoietic precursor proliferation, or factors acting on later cells in the hematopoietic pathway, including, but not limited to, hemopoietic maturation factor, thrombopoietin, stem cell factor, erythropoietin, G-CSF, GM-CSF, etc.

In an alternative embodiment, TIE-2 receptor antagonist fusion polypeptides are used to diagnose or treat patients in which the desired result is inhibition of a hematopoietic pathway, such as for the treatment of myeloproliferative or other proliferative disorders of blood forming organs

such as thrombocythemias, polycythemias and leukemias. In such embodiments, treatment may comprise use of a therapeutically effective amount of the fusion polypeptides as described herein.

Effective doses useful for treating these or other diseases or disorders may be determined using methods known to one skilled in the art [see, for example, Fingl, et al., The Pharmacological Basis of Therapeutics, Goodman and Gilman, eds. Macmillan Publishing Co., New York, pp. 1-46 ((1975)]. Pharmaceutical compositions for use according to the invention include the fusion polypeptides described above in a pharmacologically acceptable liquid, solid or semi-solid carrier, linked to a carrier or targeting molecule (e.g., antibody, hormone, growth factor, etc.) and/or incorporated into liposomes, microcapsules, and controlled release preparation prior to administration *in vivo*. For example, the pharmaceutical composition may comprise a fusion polypeptide in an aqueous solution, such as sterile water, saline, phosphate buffer or dextrose solution. Alternatively, the active agents may be comprised in a solid (e.g. wax) or semi-solid (e.g. gelatinous) formulation that may be implanted into a patient in need of such treatment. The administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an implanted device.

Administration may result in the distribution of the active agent of the invention throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of agent may be desirable. In some situations, an implant containing active agent may be placed in or near the lesioned area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

The present invention also provides for pharmaceutical compositions comprising the fusion polypeptides described herein, in a pharmacologically acceptable vehicle. The compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

EXAMPLES

Angiopoietin ligands:

As described *supra*, experiments with mutants of Ang-1 and Ang-2 have demonstrated that the fibrinogen domains (FD) are the receptor-binding domains, and that dimerized versions (dimerization occurs due to the interaction between the Fc components of adjacent molecules), for example Ang-1-FD-Fc, can bind to the Tie-2 receptor with much higher affinity than monomeric Ang-1-FD. However, Ang-1-FD-Fc is not able to induce phosphorylation (activate) the Tie-2 receptor on endothelial cells unless it is further clustered with goat anti-human Fc antibodies (Jackson Immunoresearch). For this reason, mutant versions of Ang-1-FD and Ang-2-FD were designed that were intrinsically more highly clustered.

Two general types of nucleic acid molecules were constructed. The first type consisted of two tandem copies of Ang-1-FD fused to an Fc tag, thus leading

to a secreted polypeptide molecule that is dimeric with respect to the Fc tag but tetrameric with respect to Ang-1-FD. Similarly, two tandem copies of Ang-2-FD fused to an Fc tag constituted the angiopoietin-2 version of this type of construct. These molecules were designated Ang-1-FD-FD-Fc and Ang-2-FD-FD-Fc, respectively.

In the second type of nucleic acid molecule constructed, two copies of Ang-1-FD were connected by an Fc tag bridging between them, thus creating the structure Ang-1-FD-Fc-FD that is still dimeric with respect to the Fc, as well as tetrameric with respect to Ang-1-FD. An angiopoietin-2 version was also constructed and these two molecules were designated Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD, respectively.

For either type of construct, similar properties were observed: unlike dimeric Ang-1-FD-Fc, which fails to activate Tie-2 in endothelial cells, both Ang-1-FD-FD-Fc and Ang-1-FD-Fc-FD could readily activate Tie-2 in endothelial cells, with a potency comparable to that of the native ligand. Also, like native angiopoietin-2, Ang-2-FD-Fc-FD could antagonize angiopoietin-1 activity with a potency that is comparable to that of native angiopoietin-2, and with much greater potency than the marginally antagonistic activity of the Ang-2-FD-Fc dimer.

Construction of mutant angiopoietin nucleic acid molecules.

All of the following nucleic acid molecules were constructed by standard recombinant DNA techniques (See e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), sequence-verified by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and subcloned into the mammalian expression vector pMT21 (Genetics

Institute, Inc.) with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5′ end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

Example 1: Construction of the Ang-1-FD-FD-Fc, Ang-2-FD-FD-Fc, Ang-1-FD-Fc-FD, and Ang-2-FD-Fc-FD nucleic acid molecules.

Ang-1-FD-FD-Fc: Ang-1-FD-FD-Fc consists of a trypsin signal sequence at its amino terminus to allow for secretion (bases 1-45 of Figure 1A) followed by the angiopoietin-1 fibrinogen domain (FD) (bases 46-690 of Figure 1A-Figure 1B), a short bridging sequence consisting of the amino acids Gly-Pro Ala-Pro (bases 691-702 of Figure 1B), a second angiopoietin-1 FD (bases 703-1750 of Figure 1B-Figure 1D), another bridging sequence consisting of the amino acids Gly-Pro-Gly (bases 1351-1359 of Figure 1D), and the coding sequence for the Fc portion of human IgG1 (bases 1360-2058 of Figure 1D-Figure 1E).

Ang-2-FD-FD-Fc: The Ang-2-FD-FD-Fc nucleic acid molecule was similarly constructed. It consists of a trypsin signal sequence (bases 1-45 of Figure 2A), an angiopoietin-2 FD (bases 46-690 of Figure 2A- Figure 2B), a bridging amino acid sequence Gly-Gly-Pro-Ala-Pro (bases 691-705 of Figure 2B), a second angiopoietin-2 FD (bases 706-1353 of Figure 2B-Figure 2D), another bridging amino acid sequence Gly-Pro-Gly (bases 1354-1362 of Figure 2D), and the coding sequence for the Fc portion of human IgG1 (bases 1363-2061 of Figure 2D-Figure 2E.

Ang-1-FD-Fc-FD: The Ang-1-FD-Fc-FD consists of a trypsin signal sequence (bases 1-45 of Figure 3A), an angiopoietin-1 FD (bases 46-690 of Figure 3A-3B), the bridging amino acid sequence Gly-Pro-Gly (bases 691-699 of Figure

3B), the coding sequence for the Fc portion of human IgG1 (bases 700-1395 of Figure 3B-3D), another bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1396-1419 of Figure 3D), and a second angiopoietin-1 FD (bases 1420-2067 of Figure 3D-Figure 3E).

Ang-2-FD-Fc-FD: The Ang-2-FD-Fc-FD nucleic acid molecule consists of a trypsin signal sequence (bases 1-45 of Figure 4A), an angiopoietin-2 FD domain (bases 46-690 of Figure 4A-Figure 4B), the bridging amino acid sequence Gly-Gly-Pro-Gly (bases 691-702 of Figure 4B), the coding sequence for the Fc portion of human IgG1 (bases 703-1398 of Figure 4B- Figure 4D), the bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1399-1422 of Figure 4D), and a second angiopoietin-2 FD (bases 1423-2067 of Figure 4D-Figure 4E).

Example 2: Characterization of Ang-1 FD-Fc-FD protein.

Molecular Weight Analysis: The predicted molecular weight for Ang-1-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form (with respect to the Fc) has a predicted weight of 76,349. In addition, there are three predicted N-linked glycosylation sites, approximately 2500 MW/site, that could potentially increase the molecular weight of the monomeric protein to 83,849. Due to the interaction between the Fc components of adjacent molecules, the protein actually exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,698. Subsequent SDS PAGE analyses of COS cell-derived protein described *infra* confirmed these approximate molecular weights, with a band running at about 210kD under non-reducing conditions and a band running at about 85kD under reducing conditions (Figure 5). Light scatter analysis was performed to further confirm the molecular weight and, more importantly, determine whether or not the protein was a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To

determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent was monitored with an on line light scattering detector and a refractive index and/or a UV detector. The light scattering detector is a MiniDawn laser light scattering detector was from Wyatt Technology Corporation (Santa Barbara, CA). This instrument measures static light at three different angles. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) was used to calculate the protein concentration based on either dn/dc (dn = change of refractive index; dc = concentration) or the extinction coefficient of the protein. The molecular weight of protein is then calculated from the angular dependence of light scattering. Figure 6 shows the results of this analysis using COS cell-derived protein. The molecular weight of the dimeric protein appears to be approximately 200kD and presence of a single peak implies that the protein solution is, in fact, homogenous.

<u>Expression Level in COS Cells:</u> COS cell supernatant containing recombinant Ang-1-FD-Fc-FD protein was generated by transiently transfecting COS cells with the Ang1-FD-Fc-FD DNA construct described *supra*. All transfections were performed using standard techniques known in the art. The COS cell supernatant was analyzed using Biacore technology (Pharmacia, Inc.) to quantitate the amount of Ang-1-FD-Fc-FD protein present in the supernatant. This analysis resulted in an RU value of 765, which is equivalent to 0.9mg of recombinant protein/liter of COS cell supernatant. These values represent very high levels of expression.

<u>Purification of COS Supernatants</u>: Because the Ang-1-FD-Fc-FD protein contains an Fc domain, purification is relatively simple and straight forward using standard Protein A column chromatography (Pharmacia, Inc.) followed by standard size exclusion chromatography (Pharmacia, Inc.). In fact, the relative ease of purification of the Ang-1-FD-Fc-FD protein gives it a distinct advantage over the parent protein, angiopoietin-1, from which

it is derived, and the mutant version of angiopoietin-1 called Ang1* that consists of the N-terminal of angiopoietin-2 fused to the coiled-coil domain and fibrinogen domain of angiopoietin-1 and that has a Cys to Ser mutation at amino acid 245. (See PCT International Application entitled "Modified TIE-2 Receptor Ligands," published as WO 98/05779 on 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc., especially Figure 27, which is hereby incorporated by reference).

Both angiopoietin-1 and Ang1* require extensive, expensive and labor-intensive purification schemes that result in relatively poor yields of recombinant protein. The need for cost-effective, simple purification schemes for biologicals intended for clinical use can not be over-emphasized.

The COS cell supernatant was purified as described *supra* and yielded approximately 1 mg of purified Ang-1-FD-Fc-FD protein that was used in the studies described *infra* to further characterize the protein.

<u>N-terminal sequencing of COS cell-derived Ang-1-FD-Fc-FD protein</u>: Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. This was of concern because the mutant molecule, Ang1*, has a history of containing between 10-20% N-terminally truncated species. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys. This sequence can be found at amino acids 16-20 of Figure 3A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 Figure 3A.

<u>Receptor binding analysis of COS cell-derived Ang-1-FD-Fc-FD</u>: Previous studies have determined that the fibrinogen domain (FD) of the angiopoietin molecules is necessary for ligand/receptor interaction. Furthermore, in order for high affinity binding to the Tie-2 receptor to

occur, native angiopoietin-1, angiopoietin-2, and the mutant Ang1* must form at least tetrameric, and possibly higher order, multimers. To determine whether the COS cell-derived Ang-1-FD-Fc-FD protein, which is tetrameric with respect to the FD domain, could bind to Tie-2 with high affinity, standard Biacore analysis was performed. Briefly, Tie-2-Fc receptor body protein, which is a fusion protein comprising the ectodomain of Tie-2 fused to the Fc domain of human IgG1, was immobilized on a Biacore chip. Ang-1-FD-Fc-FD-containing solution was passed over the chip and binding between Tie-2 ectodomain and Ang-1-FD-Fc-FD was allowed to occur. The binding step was followed by a 0.5 M NaCl high salt wash. The high salt wash was not able to disrupt the interaction between the Ang-1-FD-Fc-FD protein and the Tie-2 receptor ectodomain, implying that there is a strong interaction between the mutant ligand and receptor. This result is consistent with earlier Biacore results in which both Ang-1-FD-Fc-FD parent molecule, angiopoietin-1 and the mutant Ang1* molecule, have been shown to interact strongly with the Tie-2-Fc receptor and that this interaction is not disrupted by high salt. In contrast, several mutant molecules derived from the parent angiopoietin-1 molecule are readily dissociated from the Tie-2-Fc receptor when treated with high salt. The mutant molecules, designated Ang-1/FD (a monomer with respect to the FD), Ang-1/FD-Fc (also a monomer with respect to the FD, but which is able to form a dimer due to the presence of the Fc domain), and Ang-1/C/FD (a monomer with respect to the FD, but which also contains the coiled-coil domain of angiopoietin-1), do not exist in multimeric forms sufficient for high affinity binding to the Tie-2 receptor.

Example 3: Characterization of COS cell-derived Ang-2-FD-Fc-FD protein.

Molecular Weight Analysis: As described for Ang-1-FD-Fc-FD *supra*, the predicted molecular weight for Ang-2-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form of Ang-2-FD-Fc-FD has a predicted

weight of 76,052, with three predicted N-linked glycosylation sites that could potentially increase the molecular weight of the monomeric protein to 83,552. Like Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,104. SDS PAGE analyses of COS cell-derived protein confirmed these approximate molecular weights, with a band running at about 200kD under non-reducing conditions and a band running at about 88kD under reducing conditions (Figure 7). Light scatter analysis confirmed the molecular weight (171kD) and revealed that the Ang-2-FD-Fc-FD protein, like Ang-1-FD-Fc-FD, exists as a homogeneous species (Figure 8).

__Expression Level in COS Cells:__ COS cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was generated by transiently transfecting COS cells with the Ang-2-FD-Fc-FD DNA construct described *supra*. The COS cell supernatant was analyzed by Biacore to quantitate the amount of Ang-2-FD-Fc-FD protein present in the supernatant. This analysis resulted in an RU value of 606, which is equivalent to 0.7mg of recombinant protein/liter of COS cell supernatant. These values represent relatively high levels of expression.

__Purification of COS Supernatants:__ As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The COS cell supernatant was purified as described for Ang-1-FD-Fc-FD supra and yielded approximately 2 mg of purified Ang-2-FD-Fc-FD protein that was used in the studies described *infra* to further characterize this protein.

__N-terminal sequencing:__ Purified COS cell-derived Ang-2-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Glu, wherein X is Cys. This

sequence can be found at amino acids 16-20 of Figure 4A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A.

<u>Receptor binding analysis of COS cell-derived protein</u>: To determine whether the COS cell-derived Ang-2-FD-Fc-FD protein could bind to the Tie-2 receptor, standard Biacore analysis was performed as described for Ang-1-FD-Fc-FD *supra*. As with Ang-1-FD-Fc-FD, a high salt wash was not able to disrupt the interaction between the Ang-2-FD-Fc-FD protein and the Tie-2-Fc receptor, again implying that there is a strong interaction between mutant ligand and receptor.

<u>Example 4: Effects of COS cell-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.</u>

Because Ang-1-FD-Fc-FD is a mutant molecule derived from the agonist angiopoietin-1 and Ang-2-FD-Fc-FD is a mutant molecule derived from the antagonist angiopoietin-2, we wanted to determine whether or not these two mutant molecules would retain the activity associated with the parent molecule from which it was derived.

<u>Assay system</u>: All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA <u>80</u>:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill in the art.

<u>(A) Ang1*-mediated vs. Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells</u>: EAhy926 cells were stimulated with either 0.1 μg/ml, 0.2 μg/ml, or 0.8 μg/ml Ang1* or Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 9).

<u>(B) Ability of Ang-2-FD-Fc-FD to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells:</u> EAhy926 cells were treated with 0.4 μg/ml of the Tie-2 agonist Ang1* and 1 μg/ml, 2 μg/ml, 4 μg/ml. 6 μg/ ml, or 8 μg/ml of Ang-2-FD-Fc-FD. As shown in Figure 10, Ang-2-FD-Fc-FD is able to block Ang1* stimulation of the Tie-2 receptor when it is present in at least a 10-15 fold molar excess of Ang1*.

<u>(C) Ability of angiopoietin-2 to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells:</u> To compare the blocking effects of the naturally occurring antagonist angiopoietin-2 with that of Ang-2-FD-Fc-FD, the same experiment described in (B) *supra* was performed, substituting angiopoietin-2 for Ang-2-FD-Fc-FD. The results of this experiment are shown in Figure 11. At a 20 fold molar excess, the angiopoietin-2 has not reduced the phosphorylation level to 50%. This result, coupled with the results described in (B) *supra* implies that Ang-2-FD-Fc-FD is a more potent inhibitor or Ang1*-mediated Tie-2 receptor phosphorylation than angiopoietin-2.

<u>(D) Ability of Ang-2-FD-Fc-FD to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells:</u> EAhy926 cells were treated with 0.2 μg/ml of the naturally occurring Tie-2 agonist angiopoietin-1 and 1 μg/ml, 2 μg/ml, 4 μg/ml. 6 μg/ ml, or 8 μg/ml of Ang-2-FD-Fc-FD. The results of this experiment, shown in Figure 12, show that while there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, Ang-2-FD-Fc-FD seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 10 and described in (B) *supra*.

(E) <u>Ability of angiopoietin-2 to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells:</u> EAhy926 cells were treated with 0.2 μg/ml of the angiopoietin-1 and 1 μg/ml, 2 μg/ml, 4 μg/ml,

6 μg/ ml, or 8 μg/ml of angiopoietin-2. The results of this experiment, shown in Figure 13, show that there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, but, like Ang-2-FD-Fc-FD, angiopoietin-2 seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 11 and described in (C) *supra*.

__Example 5: Construction of Ang-1-FD-Fc-FD CHO cell expression vector pRG763/Ang-1-FD-Fc-FD.__

The pRG763/Ang-1-FD-Fc-FD CHO cell expression vector was constructed by isolating from the plasmid pCDNA3.1/Ang1-FD-Fc-FD a 2115 base pair EcoRI - NotI fragment containing Ang1-FD-Fc-FD and ligating this fragment into pRG763 vector digested with EcoRI and NotI. A large scale (2L) culture of E. coli DH10B cells carrying the pRG763/Ang-1-FD-Fc-FD plasmid was grown overnight in TB + ampicillin and the plasmid DNA was extracted using a Promega Wizard Plus Maxiprep kit, following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined in a UV spectrophotometer and fluorometer. The plasmid DNA was verified by digestion of aliquots with NcoI and HincII restriction enzymes. All restriction enzyme digest fragments corresponded to the predicted sizes in a 1% agarose gel.

__Example 6: Expression of Ang-1-FD-Fc-FD in CHO cells.__

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763/Ang-1-FD-Fc-FD using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10%

FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and purified as described *infra*.

Example 7: Construction of Ang-2-FD-Fc-FD CHO cell expression vector pRG763/Ang-2-FD-Fc-FD.

The plasmid pRG763/Ang-2-FD-Fc-FD was constructed by isolating from the plasmid pCDNA3.1/Ang-2-FD-Fc-FD a 2097 base pair EcoRI - NotI fragment containing Ang-2-FD-Fc-FD and ligating this fragment into the pRG763 vector digested with EcoRI and NotI. A large scale (1L) culture of E. coli DH10B cells carrying the pRG763/Ang-2-FD-Fc-FD plasmid was grown overnight in TB + ampicillin and the plasmid DNA was extracted using a Promega Wizard Plus Maxiprep kit, following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined in a UV spectrophotometer and fluorometer. The plasmid DNA was also verified by digestion of plasmid DNA with NcoI and Ppu10I restriction enzymes. All restriction enzyme digest fragments corresponded to the predicted sizes in a 1% agarose gel.

Example 8: Expression of Ang-2-FD-Fc-FD in CHO cells.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 µg of pRG763/Ang-2-FD-Fc-FD using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after

adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles purified as described infra.

<u>**Example 9: Characterization of stable CHO clone-derived Ang-1-FD-Fc-FD protein.**</u>

<u>**Molecular Weight Analysis**</u>: The predicted molecular weight for stable CHO clone-derived Ang-1-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form (with respect to the Fc) has a predicted weight of 76,349. In addition, there are three predicted N-linked glycosylation sites, approximately 2500 MW/site, that could potentially increase the molecular weight of the monomeric protein to 83,849. Due to the interaction between the Fc components of adjacent molecules, the protein actually exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,698. Subsequent SDS PAGE analyses confirmed these approximate molecular weights, with a band running at about 210kD under non-reducing conditions and a band running at about 85kD under reducing conditions. Light scatter analysis was performed to further confirm the molecular weight and, more importantly, determine whether or not the protein was a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent was monitored with an on line light scattering detector and a refractive index and/or a UV detector. The light scattering detector is a MiniDawn laser light scattering detector was from Wyatt Technology

Corporation (Santa Barbara, CA). This instrument measures static light at three different angles. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) was used to calculate the protein concentration based on either dn/dc (dn = change of refractive index; dc = concentration) or the extinction coefficient of the protein. The molecular weight of protein is then calculated from the angular dependence of light scattering. The results of this analysis show that the dimeric protein appears to be approximately 173.9kD and the presence of a single peak implies that the protein solution is homogenous.

__Expression level of Ang-1-FD-Fc-FD in stable CHO clones:__ CHO cell supernatant containing recombinant Ang-1-FD-Fc-FD protein was generated by stably transfecting CHO cells with the Ang-1-FD-Fc-FD DNA construct described *supra*. The CHO cell supernatant was analyzed by standard ELISA using an anti-human IgG antibody as a capture antibody and an anti-human IgG antibody conjugated to alkaline phosphatase as a reporter antibody to quantitate the amount of Ang-1-FD-Fc-FD protein present in the supernatant. This analysis revealed expression levels of 2-3 pg/cell/day.

__Purification of Ang-1-FD-Fc-FD protein derived from stable CHO clone supernatants__: Because the Ang-1-FD-Fc-FD protein contains an Fc domain, purification is relatively simple and straight forward using standard Protein A column chromatography (Pharmacia, Inc.) followed by standard size exclusion chromatography (Pharmacia, Inc.). The CHO cell supernatant was purified as described *supra* and the purified ANG-1-FD-Fc-FD protein was used in the studies described *infra* to further characterize the protein.

__N-terminal sequencing of stable CHO clone-derived Ang-1-FD-Fc-FD protein__: Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal

sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys. This sequence can be found at amino acids 16-20 of Figure 3A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 Figure 3A.

Example 10: Characterization of stable CHO clone-derived Ang-2-FD-Fc-FD protein.

Molecular Weight Analysis: As described for stable CHO clone-derived Ang-1-FD-Fc-FD *supra*, the predicted molecular weight for stable CHO clone-derived Ang-2-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT). The monomeric form of Ang-2-FD-Fc-FD has a predicted weight of 76,052, with three predicted N-linked glycosylation sites that could potentially increase the molecular weight of the monomeric protein to 83,552. Like Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,104. SDS PAGE analyses confirmed these approximate molecular weights, with a band running at about 200kD under non-reducing conditions and a band running at about 85kD under reducing conditions. Light scatter analysis confirmed the molecular weight (176.6kD) and revealed that the stable CHO clone-derived Ang-2-FD-Fc-FD protein, like stable CHO clone-derived Ang-1-FD-Fc-FD, exists as a homogeneous species.

Expression level of Ang-2-FD-Fc-FD derived from stable CHO clones: CHO cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was generated by stably transfecting CHO cells with the Ang-2-FD-Fc-FD DNA construct described *supra*. The CHO cell supernatant was analyzed by standard ELISA using an anti-human IgG antibody as a capture antibody and an anti-human IgG antibody conjugated to alkaline phosphatase as a reporter antibody to quantitate the amount of Ang-2-FD-Fc-FD protein present in the supernatant. This analysis revealed expression levels of approximately 1-2 pg/cell/day.

Purification of stable CHO clone-derived Ang-2-FD-Fc-FD from cell supernatants: As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The CHO cell supernatant was purified as described for stable CHO clone-derived Ang-1-FD-Fc-FD *supra* and was used in the studies described *infra* to further characterize this protein.

N-terminal sequencing of stable CHO clone-derived Ang-2-FD-Fc-FD protein: Purified stable CHO clone-derived Ang-2-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Asp-X-Ala-Glu-Val, wherein X is Cys. This sequence can be found at amino acids 17-21 of Figure 4A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A.

Example 11: Effects of stable CHO clone-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.

Assay system: All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA 80:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill in the art.

(A) Ang1*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells: EAhy926 cells were stimulated with 0.4 μg/ml Ang1* or 0.2 μg/ml or 0.4 μg/ml stable CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that or stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 17).

<u>(B) Ability of stable CHO clone-derived Ang-2-FD-Fc-FD to block stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells:</u> EAhy926 cells were treated with 0.2 μg/ml of the Tie-2 agonist Ang-1-FD-Fc-FD and 2 μg/ml, 4 μg/ml, 8 μg/ml or 16 μg/ml of stable CHO clone-derived Ang-2-FD-Fc-FD. As shown in Figure 18, Ang-2-FD-Fc-FD is able to block stable CHO clone-derived Ang-1-FD-Fc-FD stimulation of the Tie-2 receptor when it is present in at least a 40 fold molar excess of stable CHO clone-derived Ang-1-FD-Fc-FD.

<u>Ephrin ligands:</u>

In previous experiments (Davis et al., 1994, Science, <u>266</u>:816-819; Gale et al., 1996, Neuron <u>17</u>:9-19, Gale and Yancopoulos, 1997, Cell Tissue Research <u>290</u>:227-241), soluble, unclustered Ephrin-B1-Fc and Ephrin-B2-Fc, which dimerize at their respective Fc domains and therefore are dimeric with respect to either the Ephrin-B1 or Ephrin-B2 ectodomain, failed to induce EphB2 receptor phosphorylation. However, when either molecule was multimerized by pre-clustering with an anti-Fc antibody, they became potent agonists for the EphB2 receptor, as judged by tyrosine phosphorylation of the EphB2 receptor in a COS cell reporter assay. Because multimerization of both Ephrin-B1 and Ephrin-B2 appears to be necessary for induction of receptor phosphorylation, we theorized that a molecule that contained tandem repeats of either Ephrin-B1 or Ephrin-B2 ectodomains fused to an Fc domain, which would be dimeric with respect to the Fc domain but which would be tetrameric with respect to Ephrin ectodomains, might be sufficiently clustered to induce receptor phosphorylation. To test this hypothesis, the following DNA constructs were constructed, recombinant proteins produced, and reporter assays performed.

Construction of tandem Ephrin ectodomain/Fc domain nucleic acid molecules.

All of the following nucleic acid molecules were constructed by standard recombinant DNA techniques (See e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), sequence-verified by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and subcloned into either the mammalian expression pJFE14 (Ephrin-B1-Ephrin-B1-Fc) or pMT21 (Ephrin-B2-Ephrin-B2-Fc), each with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5′ end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

Example 12: Construction of Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules.

(A) Ephrin-B1-Ephrin-B1-Fc: The Ephrin-B1-Ephrin-B1-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B1 (Davis et al., ibid.), which corresponds to nucleotides 1-711 of Figure 14A-Figure 14B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 712-720 of Figure 14B), followed by a second copy of the ectodomain of Ephrin-B1 (corresponding to nucleotides 721-1344 of Figure 14B-Figure 14D), except that in this copy of the Ephrin-B1 ectodomain the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1345-1353 of Figure 14D),

followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1354-2049 of Figure 14D-Figure 14E).

<u>(B) Ephrin-B2-Ephrin-B2-Fc:</u> The Ephrin-B2-Ephrin-B2-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann et al., 1995, Mol. Cell Biol. <u>15</u>:4821-4929), which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E).

As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.

<u>Example 13: Expression of tandem Ephrin recombinant proteins in COS cells.</u>

COS cells were transiently transfected with either the Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules described *supra* using standard transfection techniques known in the art. Two days subsequent to transfection, the growth medium (DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% calf serum) was aspirated and replaced with serum-free medium (DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine). Cell were grown for an additional three days and then the serum-free medium containing the recombinant proteins was collected. Recombinant protein concentration was determined by performing dot blots and comparing the

signal obtained to a standard curve. Once approximate protein concentrations were determined, the Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc recombinant proteins were used in the cell reporter assays described *infra*.

Example 14: Characterization of the COS cell-derived tandem Ephrin ectodomain/Fc domain recombinant proteins.

Reporter Assay: COS cells, which endogenously express the Eph family receptor EphB2 (Gale et al., 1996, Neuron 17:9-19), were used in reporter assays to evaluate the ability of Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc to induce receptor phosphorylation. The assays were performed as previously described (Davis et al., ibid.; Gale et al., ibid.). Briefly, COS cells were grown to 80-90% confluency in standard growth medium described *supra*. After growth, the medium was aspirated, and replaced with serum-free medium (described *supra*) for 1-2 hours prior to treatment with either Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc recombinant protein. The cells were stimulated with 500 ng/ml Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc for 30 minutes at 37^{o}C, with or without affinity purified human IgG1 Fc-specific goat anti-human antibody (Jackson Immunoresearch, West Grove, PA) at a final concentration of 17 µg/ml. This antibody is capable of clustering the Fc tagged fusion. Subsequent to treatment, the COS cells were harvested and cell lysates were prepared as described in Davis, et al. and Gale, et al., *supra*. The EphB2 receptor protein was immunoprecipitated from the cell lysates using an anti-EphB2 antisera (Henkemeyer et al., 1994, Oncogene 9:1001-1014). Immunoprecipitates were resolved by standard SDS PAGE and transferred to PVDF membranes (Millipore) for western blot analysis. The membranes were probed with either anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Institute, Lake Placid, NY) or anti-EphB2 antibodies (Henkemeyer, et al., ibid.) to determine the extent of EphB2

phosphorylation and the relative quantities of EphB2 in the experimental conditions described *supra*.

Results: Both Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc were shown to be approximately as active as anti-Fc antibody-clustered Ephrin-B1-Fc in their ability to induce EphB2 receptor phosphorylation in the COS cell reporter assay. Furthermore, if either of the proteins were further clustered with the goat anti-human Fc antibody, they became even more potent in their ability to induce EphB2 receptor phosphorylation. Figure 16 shows the results of this phosphorylation assay.

Example 15: Construction of Ephrin-B2-Ephrin-B2-Fc CHO expression vector.

The Ephrin-B2-Ephrin-B2-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann et al., 1995, Mol. Cell Biol. 15:4821-4929), which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E). This molecule was subcloned into the HindIII and NotI polylinker sites in the expression vector pRG763 and was designated pRG763-m(Ephrin-B2)2-Fc. As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.

Example 16: Expression of Ephrin-B2-Ephrin-B2-Fc in CHO-K1 (E1A) cells.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763-m(Ephrin-B2)2-Fc using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and purified as described *supra*.

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of the receptor binding domain of a ligand, the first subunit being fused to the N-terminal end of a multimerizing component, said multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of a ligand.

2. The nucleic acid of claim 1, wherein the receptor binding domains of the first and second subunits are copies of the receptor binding domain of the same ligand.

3. The nucleic acid of claim 1, wherein the receptor binding domains of the first subunit are copies of the receptor binding domain of a different ligand from the receptor binding domains of the second subunit.

4. The nucleic acid of claim 2, wherein the first and second subunits each have one copy of the receptor binding domain of the ligand.

5. The nucleic acid of claim 3, wherein the first and second subunits each have one copy of the receptor binding domain of the ligand.

6. The nucleic acid of claim 2, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

7. The nucleic acid of claim 4, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

8. The nucleic acid of claim 2, wherein the ligand is selected from the group consisting of the EPH family of ligands.

9. The nucleic acid of claim 4, wherein the ligand is selected from the group consisting of the EPH family of ligands.

10. The nucleic acid of claims 1 through 9, wherein the multimerizing component comprises an immunoglobulin derived domain.

11. The nucleic acid molecule of claim 10, wherein the immunoglobulin derived domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

12. A fusion polypeptide encoded by the nucleic acid molecule of claims 1 through 11.

13. A composition comprising a multimer of the fusion polypeptide of claim 12.

14. The composition of claim 13, wherein the multimer is a dimer.

15. A vector which comprises the nucleic acid molecule of claims 1 through 11.

16. An expression vector comprising a nucleic acid molecule of claims 1 through 11, wherein the nucleic acid molecule is operatively linked to an expression control sequence.

17. A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 16, in a suitable host cell.

18. The host-vector system of claim 17, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell or mammalian cell.

19. The host-vector system of claim 17, wherein the suitable host cell is E. coli.

20. The host-vector system of claim 17, wherein the suitable host cell is a COS cell.

21. The host-vector system of claim 17, wherein the suitable host cell is a CHO cell.

22. A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claims 17 through 21, under conditions permitting production of the fusion polypeptide and recovering the polypeptide so produced.

23. A nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises more than one copy of the receptor binding domain of a ligand in tandem, and wherein either the N-terminal or the C-terminal receptor binding domain is also fused to a multimerizing component.

24. The nucleic acid of claim 23, wherein the receptor binding domains are fused contiguously.

25. The nucleic acid of claim 23, wherein the ligand is not a member of the EPH family of ligands.

26. The nucleic acid of claim 24, wherein the ligand is not a member of

the EPH family of ligands.

27. The nucleic acid of claim 23, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

28. The nucleic acid of claim 24, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

29. The nucleic acid of claims 23 through 28, wherein the multimerizing component comprises an immunoglobulin derived domain.

30. The nucleic acid molecule of claim 29, wherein the immunoglobulin derived domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

31. A fusion polypeptide encoded by the nucleic acid molecule of claims 23 through 30.

32. A composition comprising a multimer of the fusion polypeptide of claim 31.

33. The composition of claim 32, wherein the multimer is a dimer.

34. A vector which comprises the nucleic acid molecule of claims 23 through 30.

35. An expression vector comprising a nucleic acid molecule of claims 23 through 30, wherein the nucleic acid molecule is operatively linked to an expression control sequence.

36. A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 35, in a suitable host cell.

37. The host-vector system of claim 36, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell or mammalian cell.

38. The host-vector system of claim 36, wherein the suitable host cell is E. coli.

39. The host-vector system of claim 36, wherein the suitable host cell is a COS cell.

40. The host-vector system of claim 36, wherein the suitable host cell is a CHO cell.

41. A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claims 36 through 40, under conditions permitting production of the fusion polypeptide and recovering the polypeptide so produced.

Figure 1A

```
         10        20        30        40
    *    *    *    *    *    *    *    *    *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
___a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

     50        60        70        80        90
      *    *    *    *    *    *    *    *    *
AGA GAC TGT GCA GAT GTA TAT CAA GCT GGT TTT AAT AAA AGT GGA
Arg Asp Cys Ala Asp Val Tyr Gln Ala Gly Phe Asn Lys Ser Gly>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         100       110       120       130
    *    *    *    *    *    *    *    *    *
ATC TAC ACT ATT TAT ATT AAT AAT ATG CCA GAA CCC AAA AAG GTG
Ile Tyr Thr Ile Tyr Ile Asn Asn Met Pro Glu Pro Lys Lys Val>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

     140       150       160       170       180
      *    *    *    *    *    *    *    *    *
TTT TGC AAT ATG GAT GTC AAT GGG GGA GGT TGG ACT GTA ATA CAA
Phe Cys Asn Met Asp Val Asn Gly Gly Gly Trp Thr Val Ile Gln>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         190       200       210       220
    *    *    *    *    *    *    *    *    *
CAT CGT GAA GAT GGA AGT CTA GAT TTC CAA AGA GGC TGG AAG GAA
His Arg Glu Asp Gly Ser Leu Asp Phe Gln Arg Gly Trp Lys Glu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

     230       240       250       260       270
      *    *    *    *    *    *    *    *    *
TAT AAA ATG GGT TTT GGA AAT CCC TCC GGT GAA TAT TGG CTG GGG
Tyr Lys Met Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         280       290       300       310
    *    *    *    *    *    *    *    *    *
AAT GAG TTT ATT TTT GCC ATT ACC AGT CAG AGG CAG TAC ATG CTA
Asn Glu Phe Ile Phe Ala Ile Thr Ser Gln Arg Gln Tyr Met Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

     320       330       340       350       360
      *    *    *    *    *    *    *    *    *
AGA ATT GAG TTA ATG GAC TGG GAA GGG AAC CGA GCC TAT TCA CAG
Arg Ile Glu Leu Met Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         370       380       390       400
    *    *    *    *    *    *    *    *    *
TAT GAC AGA TTC CAC ATA GGA AAT GAA AAG CAA AAC TAT AGG TTG
Tyr Asp Arg Phe His Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

     410       420       430       440       450
      *    *    *    *    *    *    *    *    *
TAT TTA AAA GGT CAC ACT GGG ACA GCA GGA AAA CAG AGC AGC CTG
Tyr Leu Lys Gly His Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>
```

Figure 1B

```
          460              470              480              490
           *        *        *        *        *        *        *        *
ATC TTA CAC GGT GCT GAT TTC AGC ACT AAA GAT GCT GAT AAT GAC
Ile Leu His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    500              510              520              530              540
      *        *        *        *        *        *        *        *
AAC TGT ATG TGC AAA TGT GCC CTC ATG TTA ACA GGA GGA TGG TGG
Asn Cys Met Cys Lys Cys Ala Leu Met Leu Thr Gly Gly Trp Trp>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

          550              560              570              580
           *        *        *        *        *        *        *        *
TTT GAT GCT TGT GGC CCC TCC AAT CTA AAT GGA ATG TTC TAT ACT
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    590              600              610              620              630
      *        *        *        *        *        *        *        *
GCG GGA CAA AAC CAT GGA AAA CTG AAT GGG ATA AAG TGG CAC TAC
Ala Gly Gln Asn His Gly Lys Leu Asn Gly Ile Lys Trp His Tyr>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

          640              650              660              670
           *        *        *        *        *        *        *        *
TTC AAA GGG CCC AGT TAC TCC TTA CGT TCC ACA ACT ATG ATG ATT
Phe Lys Gly Pro Ser Tyr Ser Leu Arg Ser Thr Thr Met Met Ile>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    680              690              700              710              720
      *        *        *        *        *        *        *        *
CGA CCT TTA GAT TTT GGC CCC GCG CCT TTT AGA GAC TGT GCA GAT
Arg Pro Leu Asp Phe>
___ANG1 FIBRINO____>
                    Gly Pro Ala Pro>
                    ___GPAP BRI____>
                                    Phe Arg Asp Cys Ala Asp>
                                    ___ANG1 FIBRINOGEN-____>

          730              740              750              760
           *        *        *        *        *        *        *        *
GTA TAT CAA GCT GGT TTT AAT AAA AGT GGA ATC TAC ACT ATT TAT
Val Tyr Gln Ala Gly Phe Asn Lys Ser Gly Ile Tyr Thr Ile Tyr>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

    770              780              790              800              810
      *        *        *        *        *        *        *        *
ATT AAT AAT ATG CCA GAA CCC AAA AAG GTG TTT TGC AAT ATG GAT
Ile Asn Asn Met Pro Glu Pro Lys Lys Val Phe Cys Asn Met Asp>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

          820              830              840              850
           *        *        *        *        *        *        *        *
GTC AAT GGG GGA GGT TGG ACT GTA ATA CAA CAT CGT GAA GAT GGA
Val Asn Gly Gly Gly Trp Thr Val Ile Gln His Arg Glu Asp Gly>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>
```

Figure 1C

```
  860         870         880         890         900
   *       *       *       *       *       *       *       *       *
AGT CTA GAT TTC CAA AGA GGC TGG AAG GAA TAT AAA ATG GGT TTT
Ser Leu Asp Phe Gln Arg Gly Trp Lys Glu Tyr Lys Met Gly Phe>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

        910         920         930         940
   *       *       *       *       *       *       *       *       *
GGA AAT CCC TCC GGT GAA TAT TGG CTG GGG AAT GAG TTT ATT TTT
Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Ile Phe>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

  950         960         970         980         990
   *       *       *       *       *       *       *       *       *
GCC ATT ACC AGT CAG AGG CAG TAC ATG CTA AGA ATT GAG TTA ATG
Ala Ile Thr Ser Gln Arg Gln Tyr Met Leu Arg Ile Glu Leu Met>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

       1000        1010        1020        1030
   *       *       *       *       *       *       *       *       *
GAC TGG GAA GGG AAC CGA GCC TAT TCA CAG TAT GAC AGA TTC CAC
Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln Tyr Asp Arg Phe His>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

 1040        1050        1060        1070        1080
   *       *       *       *       *       *       *       *       *
ATA GGA AAT GAA AAG CAA AAC TAT AGG TTG TAT TTA AAA GGT CAC
Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu Tyr Leu Lys Gly His>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

       1090        1100        1110        1120
   *       *       *       *       *       *       *       *       *
ACT GGG ACA GCA GGA AAA CAG AGC AGC CTG ATC TTA CAC GGT GCT
Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu Ile Leu His Gly Ala>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

 1130        1140        1150        1160        1170
   *       *       *       *       *       *       *       *       *
GAT TTC AGC ACT AAA GAT GCT GAT AAT GAC AAC TGT ATG TGC AAA
Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp Asn Cys Met Cys Lys>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

       1180        1190        1200        1210
   *       *       *       *       *       *       *       *       *
TGT GCC CTC ATG TTA ACA GGA GGA TGG TGG TTT GAT GCT TGT GGC
Cys Ala Leu Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

 1220        1230        1240        1250        1260
   *       *       *       *       *       *       *       *       *
CCC TCC AAT CTA AAT GGA ATG TTC TAT ACT GCG GGA CAA AAC CAT
Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr Ala Gly Gln Asn His>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>
```

Figure 1D

```
        1270        1280        1290         1300
    *    *    *     *    *    *     *    *    *
GGA AAA CTG AAT GGG ATA AAG TGG CAC TAC TTC AAA GGG CCC AGT
Gly Lys Leu Asn Gly Ile Lys Trp His Tyr Phe Lys Gly Pro Ser>
____d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

 1310        1320         1330        1340        1350
    *    *    *     *    *    *     *    *    *
TAC TCC TTA CGT TCC ACA ACT ATG ATG ATT CGA CCT TTA GAT TTT
Tyr Ser Leu Arg Ser Thr Thr Met Met Ile Arg Pro Leu Asp Phe>
____d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

        1360        1370        1380         1390
    *    *    *     *    *    *     *    *    *
GGA CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA
Gly Pro Gly>
____e___e___>
            Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro>
            ____f___f___f____FC TAG [SPLIT]_f___f___f___f___>

 1400        1410         1420        1430        1440
    *    *    *     *    *    *     *    *    *
CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC
Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu>
____f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

        1450        1460        1470         1480
    *    *    *     *    *    *     *    *    *
TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro>
____f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

 1490        1500         1510        1520        1530
    *    *    *     *    *    *     *    *    *
GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu>
____f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

        1540        1550        1560         1570
    *    *    *     *    *    *     *    *    *
GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC
Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala>
____f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

 1580        1590         1600        1610        1620
    *    *    *     *    *    *     *    *    *
AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val>
____f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

        1630        1640        1650         1660
    *    *    *     *    *    *     *    *    *
GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG
Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys>
____f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>
```

Figure 1E

```
 1670       1680          1690         1700          1710
   *          *     *     *     *     *     *     *     *
GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC
Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

        1720       1730          1740          1750
   *          *     *     *     *     *     *     *     *
GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG
Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

 1760       1770          1780         1790          1800
   *          *     *     *     *     *     *     *     *
GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG
Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

        1810       1820          1830          1840
   *          *     *     *     *     *     *     *     *
GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC
Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

 1850       1860          1870         1880          1890
   *          *     *     *     *     *     *     *     *
GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG
Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

        1900       1910          1920          1930
   *          *     *     *     *     *     *     *     *
ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

 1940       1950          1960         1970          1980
   *          *     *     *     *     *     *     *     *
AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC
Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

        1990       2000          2010          2020
   *          *     *     *     *     *     *     *     *
TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG
Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

 2030       2040          2050
   *          *     *     *     *     *
CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
___f___f___f__FC TAG [SPLIT]___f___f___f___>
```

Figure 2A

```
        10         20         30         40
 *    *    *    *    *    *    *    *    *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
___a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

  50         60         70         80         90
 *    *    *    *    *    *    *    *    *
AGA GAC TGT GCT GAA GTA TTC AAA TCA GGA CAC ACC ACA AAT GGC
Arg Asp Cys Ala Glu Val Phe Lys Ser Gly His Thr Thr Asn Gly>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

        100        110        120        130
 *    *    *    *    *    *    *    *    *
ATC TAC ACG TTA ACA TTC CCT AAT TCT ACA GAA GAG ATC AAG GCC
Ile Tyr Thr Leu Thr Phe Pro Asn Ser Thr Glu Glu Ile Lys Ala>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

 140        150        160        170        180
 *    *    *    *    *    *    *    *    *
TAC TGT GAC ATG GAA GCT GGA GGA GGC GGG TGG ACA ATT ATT CAG
Tyr Cys Asp Met Glu Ala Gly Gly Gly Gly Trp Thr Ile Ile Gln>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

        190        200        210        220
 *    *    *    *    *    *    *    *    *
CGA CGT GAG GAT GGC AGC GTT GAT TTT CAG AGG ACT TGG AAA GAA
Arg Arg Glu Asp Gly Ser Val Asp Phe Gln Arg Thr Trp Lys Glu>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

 230        240        250        260        270
 *    *    *    *    *    *    *    *    *
TAT AAA GTG GGA TTT GGT AAC CCT TCA GGA GAA TAT TGG CTG GGA
Tyr Lys Val Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

        280        290        300        310
 *    *    *    *    *    *    *    *    *
AAT GAG TTT GTT TCG CAA CTG ACT AAT CAG CAA CGC TAT GTG CTT
Asn Glu Phe Val Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val Leu>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

 320        330        340        350        360
 *    *    *    *    *    *    *    *    *
AAA ATA CAC CTT AAA GAC TGG GAA GGG AAT GAG GCT TAC TCA TTG
Lys Ile His Leu Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

        370        380        390        400
 *    *    *    *    *    *    *    *    *
TAT GAA CAT TTC TAT CTC TCA AGT GAA GAA CTC AAT TAT AGG ATT
Tyr Glu His Phe Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

 410        420        430        440        450
 *    *    *    *    *    *    *    *    *
CAC CTT AAA GGA CTT ACA GGG ACA GCC GGC AAA ATA AGC AGC ATC

His Leu Lys Gly Leu Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>
```

Figure 2B

```
         460        470        480        490
       *     *     *     *     *     *     *     *     *
AGC CAA CCA GGA AAT GAT TTT AGC ACA AAG GAT GGA GAC AAC GAC
Ser Gln Pro Gly Asn Asp Phe Ser Thr Lys Asp Gly Asp Asn Asp>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

   500        510        520        530        540
       *     *     *     *     *     *     *     *     *
AAA TGT ATT TGC AAA TGT TCA CAA ATG CTA ACA GGA GGC TGG TGG
Lys Cys Ile Cys Lys Cys Ser Gln Met Leu Thr Gly Gly Trp Trp>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         550        560        570        580
       *     *     *     *     *     *     *     *     *
TTT GAT GCA TGT GGT CCT TCC AAC TTG AAC GGA ATG TAC TAT CCA
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

   590        600        610        620        630
       *     *     *     *     *     *     *     *     *
CAG AGG CAG AAC ACA AAT AAG TTC AAC GGC ATT AAA TGG TAC TAC
Gln Arg Gln Asn Thr Asn Lys Phe Asn Gly Ile Lys Trp Tyr Tyr>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         640        650        660        670
       *     *     *     *     *     *     *     *     *
TGG AAA GGC TCA GGC TAT TCG CTC AAG GCC ACA ACC ATG ATG ATC
Trp Lys Gly Ser Gly Tyr Ser Leu Lys Ala Thr Thr Met Met Ile>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

   680        690        700        710        720
       *     *     *     *     *     *     *     *     *
CGA CCA GCA GAT TTC GGG GGC CCC GCG CCT TTC AGA GAC TGT GCT
Arg Pro Ala Asp Phe>
___ANG2 FIBRINO____>
                    Gly Gly Pro Ala Pro>
                    ___GGPAP BRIDGE____>
                                        Phe Arg Asp Cys Ala>
                                        ___ANG2 FIBRINO____>

         730        740        750        760
       *     *     *     *     *     *     *     *     *
GAA GTA TTC AAA TCA GGA CAC ACC ACA AAT GGC ATC TAC ACG TTA
Glu Val Phe Lys Ser Gly His Thr Thr Asn Gly Ile Tyr Thr Leu>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

   770        780        790        800        810
       *     *     *     *     *     *     *     *     *
ACA TTC CCT AAT TCT ACA GAA GAG ATC AAG GCC TAC TGT GAC ATG
Thr Phe Pro Asn Ser Thr Glu Glu Ile Lys Ala Tyr Cys Asp Met>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

         820        830        840        850
       *     *     *     *     *     *     *     *     *
GAA GCT GGA GGA GGC GGG TGG ACA ATT ATT CAG CGA CGT GAG GAT
Glu Ala Gly Gly Gly Gly Trp Thr Ile Ile Gln Arg Arg Glu Asp>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>
```

Figure 2C

```
 860        870         880         890         900
  *    *     *     *     *     *     *     *     *
GGC AGC GTT GAT TTT CAG AGG ACT TGG AAA GAA TAT AAA GTG GGA
Gly Ser Val Asp Phe Gln Arg Thr Trp Lys Glu Tyr Lys Val Gly>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

          910         920         930         940
  *    *     *     *     *     *     *     *     *
TTT GGT AAC CCT TCA GGA GAA TAT TGG CTG GGA AAT GAG TTT GTT
Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Val>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

 950        960         970         980         990
  *    *     *     *     *     *     *     *     *
TCG CAA CTG ACT AAT CAG CAA CGC TAT GTG CTT AAA ATA CAC CTT
Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val Leu Lys Ile His Leu>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

         1000        1010        1020        1030
  *    *     *     *     *     *     *     *     *
AAA GAC TGG GAA GGG AAT GAG GCT TAC TCA TTG TAT GAA CAT TTC
Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu Tyr Glu His Phe>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

 1040       1050        1060        1070        1080
  *    *     *     *     *     *     *     *     *
TAT CTC TCA AGT GAA GAA CTC AAT TAT AGG ATT CAC CTT AAA GGA
Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile His Leu Lys Gly>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

         1090        1100        1110        1120
  *    *     *     *     *     *     *     *     *
CTT ACA GGG ACA GCC GGC AAA ATA AGC AGC ATC AGC CAA CCA GGA
Leu Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile Ser Gln Pro Gly>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

 1130       1140        1150        1160        1170
  *    *     *     *     *     *     *     *     *
AAT GAT TTT AGC ACA AAG GAT GGA GAC AAC GAC AAA TGT ATT TGC
Asn Asp Phe Ser Thr Lys Asp Gly Asp Asn Asp Lys Cys Ile Cys>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

         1180        1190        1200        1210
  *    *     *     *     *     *     *     *     *
AAA TGT TCA CAA ATG CTA ACA GGA GGC TGG TGG TTT GAT GCA TGT
Lys Cys Ser Gln Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

 1220       1230        1240        1250        1260
  *    *     *     *     *     *     *     *     *
GGT CCT TCC AAC TTG AAC GGA ATG TAC TAT CCA CAG AGG CAG AAC
Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro Gln Arg Gln Asn>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>
```

Figure 2D

```
        1270        1280        1290         1300
      *     *    *     *     *    *     *     *    *
ACA AAT AAG TTC AAC GGC ATT AAA TGG TAC TAC TGG AAA GGC TCA
Thr Asn Lys Phe Asn Gly Ile Lys Trp Tyr Tyr Trp Lys Gly Ser>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

 1310        1320        1330        1340        1350
      *     *    *     *     *    *     *     *    *
GGC TAT TCG CTC AAG GCC ACA ACC ATG ATG ATC CGA CCA GCA GAT
Gly Tyr Ser Leu Lys Ala Thr Thr Met Met Ile Arg Pro Ala Asp>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

        1360        1370        1380         1390
      *     *    *     *     *    *     *     *    *
TTC GGA CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC
Phe>
___>
    Gly Pro Gly>
    ____e___e___>
             Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys>
             ___f___f___f___f__FC TAG___f___f___f___f___>

 1400        1410        1420        1430        1440
      *     *    *     *     *    *     *     *    *
CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

        1450        1460        1470         1480
      *     *    *     *     *    *     *     *    *
CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

 1490        1500        1510        1520        1530
      *     *    *     *     *    *     *     *    *
CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT
Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

        1540        1550        1560         1570
      *     *    *     *     *    *     *     *    *
GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

 1580        1590        1600        1610        1620
      *     *    *     *     *    *     *     *    *
GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT
Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

        1630        1640        1650         1660
      *     *    *     *     *    *     *     *    *
GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC
Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>
```

Figure 2E

```
   1670      1680       1690       1700       1710
    *     *    *     *     *     *     *     *     *
AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC
Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

         1720       1730       1740       1750
    *     *    *     *     *     *     *     *     *
ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

   1760      1770       1780       1790       1800
    *     *    *     *     *     *     *     *     *
CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC
Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

         1810       1820       1830       1840
    *     *    *     *     *     *     *     *     *
CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC
Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

   1850      1860       1870       1880       1890
    *     *    *     *     *     *     *     *     *
ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

         1900       1910       1920       1930
    *     *    *     *     *     *     *     *     *
AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC
Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

   1940      1950       1960       1970       1980
    *     *    *     *     *     *     *     *     *
TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC
Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

         1990       2000       2010       2020
    *     *    *     *     *     *     *     *     *
GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

   2030      2040       2050       2060
    *     *    *     *     *     *     *
ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
___f___f___f___f_____FC TAG_f___f___f___f___f___>
```

Figure 3A

```
            10          20          30          40
       *     *     *     *     *     *     *     *     *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
___a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

     50          60          70          80          90
      *     *     *     *     *     *     *     *     *
AGA GAC TGT GCA GAT GTA TAT CAA GCT GGT TTT AAT AAA AGT GGA
Arg Asp Cys Ala Asp Val Tyr Gln Ala Gly Phe Asn Lys Ser Gly>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

           100         110         120         130
       *     *     *     *     *     *     *     *     *
ATC TAC ACT ATT TAT ATT AAT AAT ATG CCA GAA CCC AAA AAG GTG
Ile Tyr Thr Ile Tyr Ile Asn Asn Met Pro Glu Pro Lys Lys Val>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    140         150         160         170         180
      *     *     *     *     *     *     *     *     *
TTT TGC AAT ATG GAT GTC AAT GGG GGA GGT TGG ACT GTA ATA CAA
Phe Cys Asn Met Asp Val Asn Gly Gly Gly Trp Thr Val Ile Gln>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

           190         200         210         220
       *     *     *     *     *     *     *     *     *
CAT CGT GAA GAT GGA AGT CTA GAT TTC CAA AGA GGC TGG AAG GAA
His Arg Glu Asp Gly Ser Leu Asp Phe Gln Arg Gly Trp Lys Glu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    230         240         250         260         270
      *     *     *     *     *     *     *     *     *
TAT AAA ATG GGT TTT GGA AAT CCC TCC GGT GAA TAT TGG CTG GGG
Tyr Lys Met Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

           280         290         300         310
       *     *     *     *     *     *     *     *     *
AAT GAG TTT ATT TTT GCC ATT ACC AGT CAG AGG CAG TAC ATG CTA
Asn Glu Phe Ile Phe Ala Ile Thr Ser Gln Arg Gln Tyr Met Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    320         330         340         350         360
      *     *     *     *     *     *     *     *     *
AGA ATT GAG TTA ATG GAC TGG GAA GGG AAC CGA GCC TAT TCA CAG
Arg Ile Glu Leu Met Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

           370         380         390         400
       *     *     *     *     *     *     *     *     *
TAT GAC AGA TTC CAC ATA GGA AAT GAA AAG CAA AAC TAT AGG TTG
Tyr Asp Arg Phe His Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>
```

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Figure 3B

```
  410        420        430        440        450
   *    *    *    *    *    *    *    *    *
TAT TTA AAA GGT CAC ACT GGG ACA GCA GGA AAA CAG AGC AGC CTG
Tyr Leu Lys Gly His Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       460        470        480        490
   *    *    *    *    *    *    *    *    *
ATC TTA CAC GGT GCT GAT TTC AGC ACT AAA GAT GCT GAT AAT GAC
Ile Leu His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

  500        510        520        530        540
   *    *    *    *    *    *    *    *    *
AAC TGT ATG TGC AAA TGT GCC CTC ATG TTA ACA GGA GGA TGG TGG
Asn Cys Met Cys Lys Cys Ala Leu Met Leu Thr Gly Gly Trp Trp>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       550        560        570        580
   *    *    *    *    *    *    *    *    *
TTT GAT GCT TGT GGC CCC TCC AAT CTA AAT GGA ATG TTC TAT ACT
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

  590        600        610        620        630
   *    *    *    *    *    *    *    *    *
GCG GGA CAA AAC CAT GGA AAA CTG AAT GGG ATA AAG TGG CAC TAC
Ala Gly Gln Asn His Gly Lys Leu Asn Gly Ile Lys Trp His Tyr>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       640        650        660        670
   *    *    *    *    *    *    *    *    *
TTC AAA GGG CCA AGT TAC TCC TTA CGT TCC ACA ACT ATG ATG ATT
Phe Lys Gly Pro Ser Tyr Ser Leu Arg Ser Thr Thr Met Met Ile>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

  680        690        700        710        720
   *    *    *    *    *    *    *    *    *
CGA CCT TTA GAT TTT GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA
Arg Pro Leu Asp Phe>
___ANG1 FIBRINO____>
                    Gly Pro Gly>
                    ___c___c___>
                                Glu Pro Lys Ser Cys Asp Lys>
                                ___d___d__FC TAG___d___d___>

       730        740        750        760
   *    *    *    *    *    *    *    *    *
ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA
Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  770        780        790        800        810
   *    *    *    *    *    *    *    *    *
CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG
Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>
```

Figure 3C

```
           820          830          840          850
      *      *      *      *      *      *      *      *      *
ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

 860          870          880          890          900
      *      *      *      *      *      *      *      *      *
CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

           910          920          930          940
      *      *      *      *      *      *      *      *      *
GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC
Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

 950          960          970          980          990
      *      *      *      *      *      *      *      *      *
AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC
Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

          1000         1010         1020         1030
      *      *      *      *      *      *      *      *      *
TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

1040         1050         1060         1070         1080
      *      *      *      *      *      *      *      *      *
CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG
Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

          1090         1100         1110         1120
      *      *      *      *      *      *      *      *      *
CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG
Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

1130         1140         1150         1160         1170
      *      *      *      *      *      *      *      *      *
CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

          1180         1190         1200         1210
      *      *      *      *      *      *      *      *      *
TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG
Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

1220         1230         1240         1250         1260
      *      *      *      *      *      *      *      *      *
GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC
Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>
```

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Figure 3D

```
        1270         1280         1290         1300
          *      *      *      *      *      *      *      *      *
TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1310         1320         1330         1340         1350
          *      *      *      *      *      *      *      *      *
CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

        1360         1370         1380         1390
          *      *      *      *      *      *      *      *      *
CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA
His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1400         1410         1420         1430         1440
          *      *      *      *      *      *      *      *      *
GGC GGT GGC GGT TCT GGC GCG CCT TTT AGA GAC TGT GCA GAT GTA
Gly Gly Gly Gly Ser Gly Ala Pro>
___G4S LINKER/ASC BRIDGE (N____>
                                Phe Arg Asp Cys Ala Asp Val>
                                ___ANG1 FIBRINOGEN-LIKE____>

        1450         1460         1470         1480
          *      *      *      *      *      *      *      *      *
TAT CAA GCT GGT TTT AAT AAA AGT GGA ATC TAC ACT ATT TAT ATT
Tyr Gln Ala Gly Phe Asn Lys Ser Gly Ile Tyr Thr Ile Tyr Ile>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

  1490         1500         1510         1520         1530
          *      *      *      *      *      *      *      *      *
AAT AAT ATG CCA GAA CCC AAA AAG GTG TTT TGC AAT ATG GAT GTC
Asn Asn Met Pro Glu Pro Lys Lys Val Phe Cys Asn Met Asp Val>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

        1540         1550         1560         1570
          *      *      *      *      *      *      *      *      *
AAT GGG GGA GGT TGG ACT GTA ATA CAA CAT CGT GAA GAT GGA AGT
Asn Gly Gly Gly Trp Thr Val Ile Gln His Arg Glu Asp Gly Ser>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

  1580         1590         1600         1610         1620
          *      *      *      *      *      *      *      *      *
CTA GAT TTC CAA AGA GGC TGG AAG GAA TAT AAA ATG GGT TTT GGA
Leu Asp Phe Gln Arg Gly Trp Lys Glu Tyr Lys Met Gly Phe Gly>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

        1630         1640         1650         1660
          *      *      *      *      *      *      *      *      *
AAT CCC TCC GGT GAA TAT TGG CTG GGG AAT GAG TTT ATT TTT GCC
Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Ile Phe Ala>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>
```

Figure 3E

```
 1670        1680         1690        1700        1710
    *     *     *     *     *     *     *     *     *
ATT ACC AGT CAG AGG CAG TAC ATG CTA AGA ATT GAG TTA ATG GAC
Ile Thr Ser Gln Arg Gln Tyr Met Leu Arg Ile Glu Leu Met Asp>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

       1720        1730        1740        1750
    *     *     *     *     *     *     *     *     *
TGG GAA GGG AAC CGA GCC TAT TCA CAG TAT GAC AGA TTC CAC ATA
Trp Glu Gly Asn Arg Ala Tyr Ser Gln Tyr Asp Arg Phe His Ile>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

 1760        1770         1780        1790        1800
    *     *     *     *     *     *     *     *     *
GGA AAT GAA AAG CAA AAC TAT AGG TTG TAT TTA AAA GGT CAC ACT
Gly Asn Glu Lys Gln Asn Tyr Arg Leu Tyr Leu Lys Gly His Thr>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

       1810        1820        1830        1840
    *     *     *     *     *     *     *     *     *
GGG ACA GCA GGA AAA CAG AGC AGC CTG ATC TTA CAC GGT GCT GAT
Gly Thr Ala Gly Lys Gln Ser Ser Leu Ile Leu His Gly Ala Asp>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

 1850        1860         1870        1880        1890
    *     *     *     *     *     *     *     *     *
TTC AGC ACT AAA GAT GCT GAT AAT GAC AAC TGT ATG TGC AAA TGT
Phe Ser Thr Lys Asp Ala Asp Asn Asp Asn Cys Met Cys Lys Cys>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

       1900        1910        1920        1930
    *     *     *     *     *     *     *     *     *
GCC CTC ATG TTA ACA GGA GGA TGG TGG TTT GAT GCT TGT GGC CCC
Ala Leu Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly Pro>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

 1940        1950         1960        1970        1980
    *     *     *     *     *     *     *     *     *
TCC AAT CTA AAT GGA ATG TTC TAT ACT GCG GGA CAA AAC CAT GGA
Ser Asn Leu Asn Gly Met Phe Tyr Thr Ala Gly Gln Asn His Gly>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

       1990        2000        2010        2020
    *     *     *     *     *     *     *     *     *
AAA CTG AAT GGG ATA AAG TGG CAC TAC TTC AAA GGG CCA AGT TAC
Lys Leu Asn Gly Ile Lys Trp His Tyr Phe Lys Gly Pro Ser Tyr>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

 2030        2040         2050        2060
    *     *     *     *     *     *     *     *
TCC TTA CGT TCC ACA ACT ATG ATG ATT CGA CCT TTA GAT TTT
Ser Leu Arg Ser Thr Thr Met Met Ile Arg Pro Leu Asp Phe>
___f___f___f_ANG1 FIBRINOGEN-LIKE DOMAIN___f___f___f___>
```

Figure 4A

```
            10                  20                  30                  40
         *        *         *         *         *         *         *         *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
___a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

      50                  60                  70                  80                  90
       *         *         *         *         *         *         *         *         *
AGA GAC TGT GCT GAA GTA TTC AAA TCA GGA CAC ACC ACA AAT GGC
Arg Asp Cys Ala Glu Val Phe Lys Ser Gly His Thr Thr Asn Gly>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

            100                 110                 120                 130
         *         *         *         *         *         *         *         *
ATC TAC ACG TTA ACA TTC CCT AAT TCT ACA GAA GAG ATC AAG GCC
Ile Tyr Thr Leu Thr Phe Pro Asn Ser Thr Glu Glu Ile Lys Ala>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

      140                 150                 160                 170                 180
       *         *         *         *         *         *         *         *         *
TAC TGT GAC ATG GAA GCT GGA GGA GGC GGG TGG ACA ATT ATT CAG
Tyr Cys Asp Met Glu Ala Gly Gly Gly Gly Trp Thr Ile Ile Gln>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

            190                 200                 210                 220
         *         *         *         *         *         *         *         *
CGA CGT GAG GAT GGC AGC GTT GAT TTT CAG AGG ACT TGG AAA GAA
Arg Arg Glu Asp Gly Ser Val Asp Phe Gln Arg Thr Trp Lys Glu>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

      230                 240                 250                 260                 270
       *         *         *         *         *         *         *         *         *
TAT AAA GTG GGA TTT GGT AAC CCT TCA GGA GAA TAT TGG CTG GGA
Tyr Lys Val Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

            280                 290                 300                 310
         *         *         *         *         *         *         *         *
AAT GAG TTT GTT TCG CAA CTG ACT AAT CAG CAA CGC TAT GTG CTT
Asn Glu Phe Val Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val Leu>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

      320                 330                 340                 350                 360
       *         *         *         *         *         *         *         *         *
AAA ATA CAC CTT AAA GAC TGG GAA GGG AAT GAG GCT TAC TCA TTG
Lys Ile His Leu Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

            370                 380                 390                 400
         *         *         *         *         *         *         *         *
TAT GAA CAT TTC TAT CTC TCA AGT GAA GAA CTC AAT TAT AGG ATT
Tyr Glu His Phe Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>
```

Figure 4B

```
 410         420         430         440         450
   *     *     *     *     *     *     *     *     *
CAC CTT AAA GGA CTT ACA GGG ACA GCC GGC AAA ATA AGC AGC ATC
His Leu Lys Gly Leu Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       460         470         480         490
   *     *     *     *     *     *     *     *     *
AGC CAA CCA GGA AAT GAT TTT AGC ACA AAG GAT GGA GAC AAC GAC
Ser Gln Pro Gly Asn Asp Phe Ser Thr Lys Asp Gly Asp Asn Asp>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

 500         510         520         530         540
   *     *     *     *     *     *     *     *     *
AAA TGT ATT TGC AAA TGT TCA CAA ATG CTA ACA GGA GGC TGG TGG
Lys Cys Ile Cys Lys Cys Ser Gln Met Leu Thr Gly Gly Trp Trp>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       550         560         570         580
   *     *     *     *     *     *     *     *     *
TTT GAT GCA TGT GGT CCT TCC AAC TTG AAC GGA ATG TAC TAT CCA
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

 590         600         610         620         630
   *     *     *     *     *     *     *     *     *
CAG AGG CAG AAC ACA AAT AAG TTC AAC GGC ATT AAA TGG TAC TAC
Gln Arg Gln Asn Thr Asn Lys Phe Asn Gly Ile Lys Trp Tyr Tyr>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       640         650         660         670
   *     *     *     *     *     *     *     *     *
TGG AAA GGC TCA GGC TAT TCG CTC AAG GCC ACA ACC ATG ATG ATC
Trp Lys Gly Ser Gly Tyr Ser Leu Lys Ala Thr Thr Met Met Ile>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

 680         690         700         710         720
   *     *     *     *     *     *     *     *     *
CGA CCA GCA GAT TTC GGG GGC CCG GGC GAG CCC AAA TCT TGT GAC
Arg Pro Ala Asp Phe>
___ANG2 FIBRINO____>
                    Gly Gly Pro Gly>
                    ___GGPG BRI____>
                                    Glu Pro Lys Ser Cys Asp>
                                    ___d____FC TAG_d___d___>

       730         740         750         760
   *     *     *     *     *     *     *     *     *
AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG
Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly>
___d___d___d___d___d___d___FC TAG___d___d___d___d___d___d___>

 770         780         790         800         810
   *     *     *     *     *     *     *     *     *
GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC
Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu>
___d___d___d___d___d___d___FC TAG___d___d___d___d___d___d___>
```

Figure 4C

```
             820         830         840         850
        *     *     *     *     *     *     *     *     *
ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

     860         870         880         890         900
        *     *     *     *     *     *     *     *     *
AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC
Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

             910         920         930         940
        *     *     *     *     *     *     *     *     *
GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC
Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

     950         960         970         980         990
        *     *     *     *     *     *     *     *     *
AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

            1000        1010        1020        1030
        *     *     *     *     *     *     *     *     *
GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA
Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

    1040        1050        1060        1070        1080
        *     *     *     *     *     *     *     *     *
GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

            1090        1100        1110        1120
        *     *     *     *     *     *     *     *     *
CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

    1130        1140        1150        1160        1170
        *     *     *     *     *     *     *     *     *
GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC
Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

            1180        1190        1200        1210
        *     *     *     *     *     *     *     *     *
TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG
Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

    1220        1230        1240        1250        1260
        *     *     *     *     *     *     *     *     *
CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>
```

Figure 4D

```
        1270        1280        1290        1300
         *     *     *     *     *     *     *     *     *
GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

 1310        1320        1330        1340        1350
   *     *     *     *     *     *     *     *     *
TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT
Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

        1360        1370        1380        1390
         *     *     *     *     *     *     *     *     *
CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

 1400        1410        1420        1430        1440
   *     *     *     *     *     *     *     *     *
AAA GGC GGT GGC GGT TCT GGC GCG CCT AGA GAC TGT GCT GAA GTA
Lys>
___>
    Gly Gly Gly Gly Ser Gly Ala Pro>
    ___e___GGGGSGAP BRIDGE_e___e___>
                                    Arg Asp Cys Ala Glu Val>
                                    ___ANG2 FIBRINOGEN-____>

        1450        1460        1470        1480
         *     *     *     *     *     *     *     *     *
TTC AAA TCA GGA CAC ACC ACA AAT GGC ATC TAC ACG TTA ACA TTC
Phe Lys Ser Gly His Thr Thr Asn Gly Ile Tyr Thr Leu Thr Phe>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

 1490        1500        1510        1520        1530
   *     *     *     *     *     *     *     *     *
CCT AAT TCT ACA GAA GAG ATC AAG GCC TAC TGT GAC ATG GAA GCT
Pro Asn Ser Thr Glu Glu Ile Lys Ala Tyr Cys Asp Met Glu Ala>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

        1540        1550        1560        1570
         *     *     *     *     *     *     *     *     *
GGA GGA GGC GGG TGG ACA ATT ATT CAG CGA CGT GAG GAT GGC AGC
Gly Gly Gly Gly Trp Thr Ile Ile Gln Arg Arg Glu Asp Gly Ser>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

 1580        1590        1600        1610        1620
   *     *     *     *     *     *     *     *     *
GTT GAT TTT CAG AGG ACT TGG AAA GAA TAT AAA GTG GGA TTT GGT
Val Asp Phe Gln Arg Thr Trp Lys Glu Tyr Lys Val Gly Phe Gly>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

        1630        1640        1650        1660
         *     *     *     *     *     *     *     *     *
AAC CCT TCA GGA GAA TAT TGG CTG GGA AAT GAG TTT GTT TCG CAA
Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Val Ser Gln>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>
```

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Figure 4E

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 1670              1680          1690          1700          1710
    *        *        *        *        *        *        *        *        *
CTG ACT AAT CAG CAA CGC TAT GTG CTT AAA ATA CAC CTT AAA GAC
Leu Thr Asn Gln Gln Arg Tyr Val Leu Lys Ile His Leu Lys Asp>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

          1720          1730          1740          1750
    *        *        *        *        *        *        *        *        *
TGG GAA GGG AAT GAG GCT TAC TCA TTG TAT GAA CAT TTC TAT CTC
Trp Glu Gly Asn Glu Ala Tyr Ser Leu Tyr Glu His Phe Tyr Leu>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 1760              1770          1780          1790          1800
    *        *        *        *        *        *        *        *        *
TCA AGT GAA GAA CTC AAT TAT AGG ATT CAC CTT AAA GGA CTT ACA
Ser Ser Glu Glu Leu Asn Tyr Arg Ile His Leu Lys Gly Leu Thr>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

          1810          1820          1830          1840
    *        *        *        *        *        *        *        *        *
GGG ACA GCC GGC AAA ATA AGC AGC ATC AGC CAA CCA GGA AAT GAT
Gly Thr Ala Gly Lys Ile Ser Ser Ile Ser Gln Pro Gly Asn Asp>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 1850              1860          1870          1880          1890
    *        *        *        *        *        *        *        *        *
TTT AGC ACA AAG GAT GGA GAC AAC GAC AAA TGT ATT TGC AAA TGT
Phe Ser Thr Lys Asp Gly Asp Asn Asp Lys Cys Ile Cys Lys Cys>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

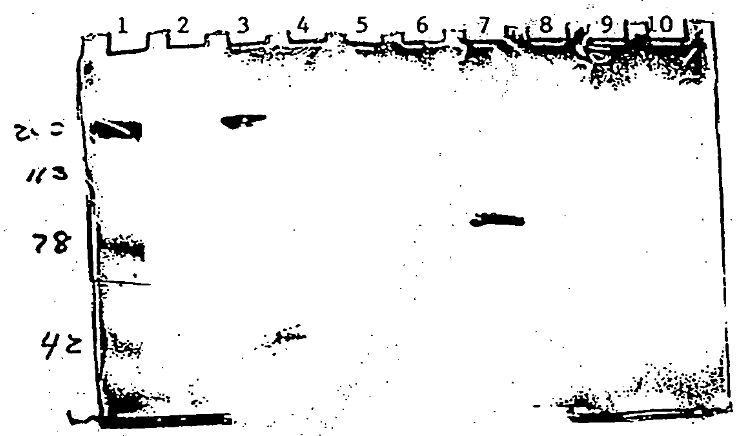
          1900          1910          1920          1930
    *        *        *        *        *        *        *        *        *
TCA CAA ATG CTA ACA GGA GGC TGG TGG TTT GAT GCA TGT GGT CCT
Ser Gln Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly Pro>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 1940              1950          1960          1970          1980
    *        *        *        *        *        *        *        *        *
TCC AAC TTG AAC GGA ATG TAC TAT CCA CAG AGG CAG AAC ACA AAT
Ser Asn Leu Asn Gly Met Tyr Tyr Pro Gln Arg Gln Asn Thr Asn>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

          1990          2000          2010          2020
    *        *        *        *        *        *        *        *        *
AAG TTC AAC GGC ATT AAA TGG TAC TAC TGG AAA GGC TCA GGC TAT
Lys Phe Asn Gly Ile Lys Trp Tyr Tyr Trp Lys Gly Ser Gly Tyr>
____f____f____f____ANG2 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 2030              2040          2050          2060          2070
    *        *        *        *        *        *        *        *        *
TCG CTC AAG GCC ACA ACC ATG ATG ATC CGA CCA GCA GAT TTC TGA
Ser Leu Lys Ala Thr Thr Met Met Ile Arg Pro Ala Asp Phe>
____f____f____f_ANG2 FIBRINOGEN-LIKE DOMAIN____f____f____f____>
```

Figure 5



Ang1-FD-Fc-FD

Figure 6

Molar Mass vs. Volume

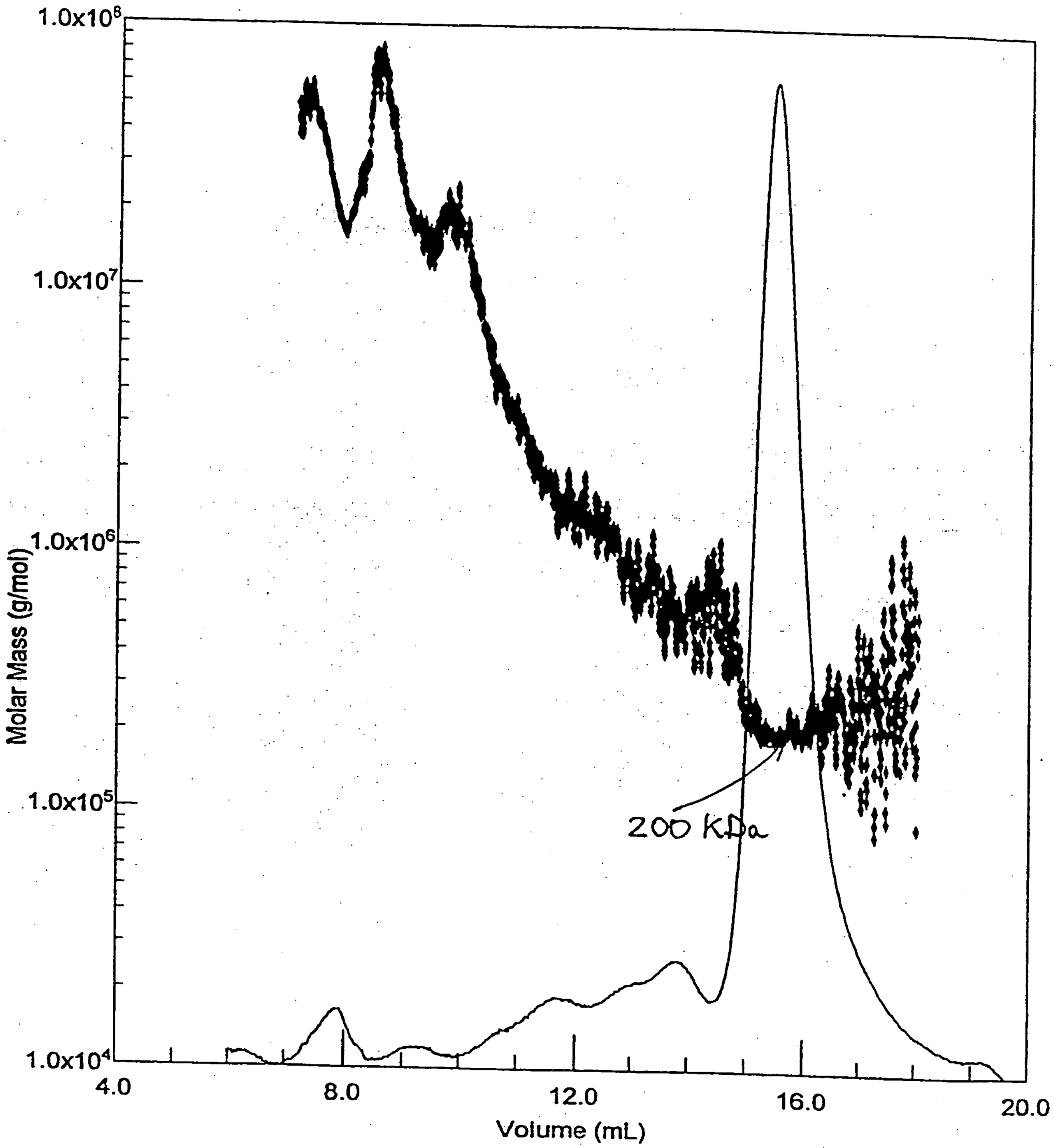
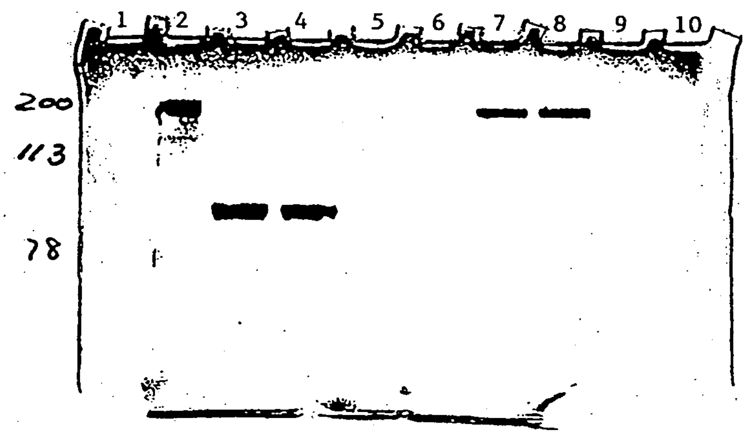


Figure 7



Ang2-FD-Fc-FD

Figure 8

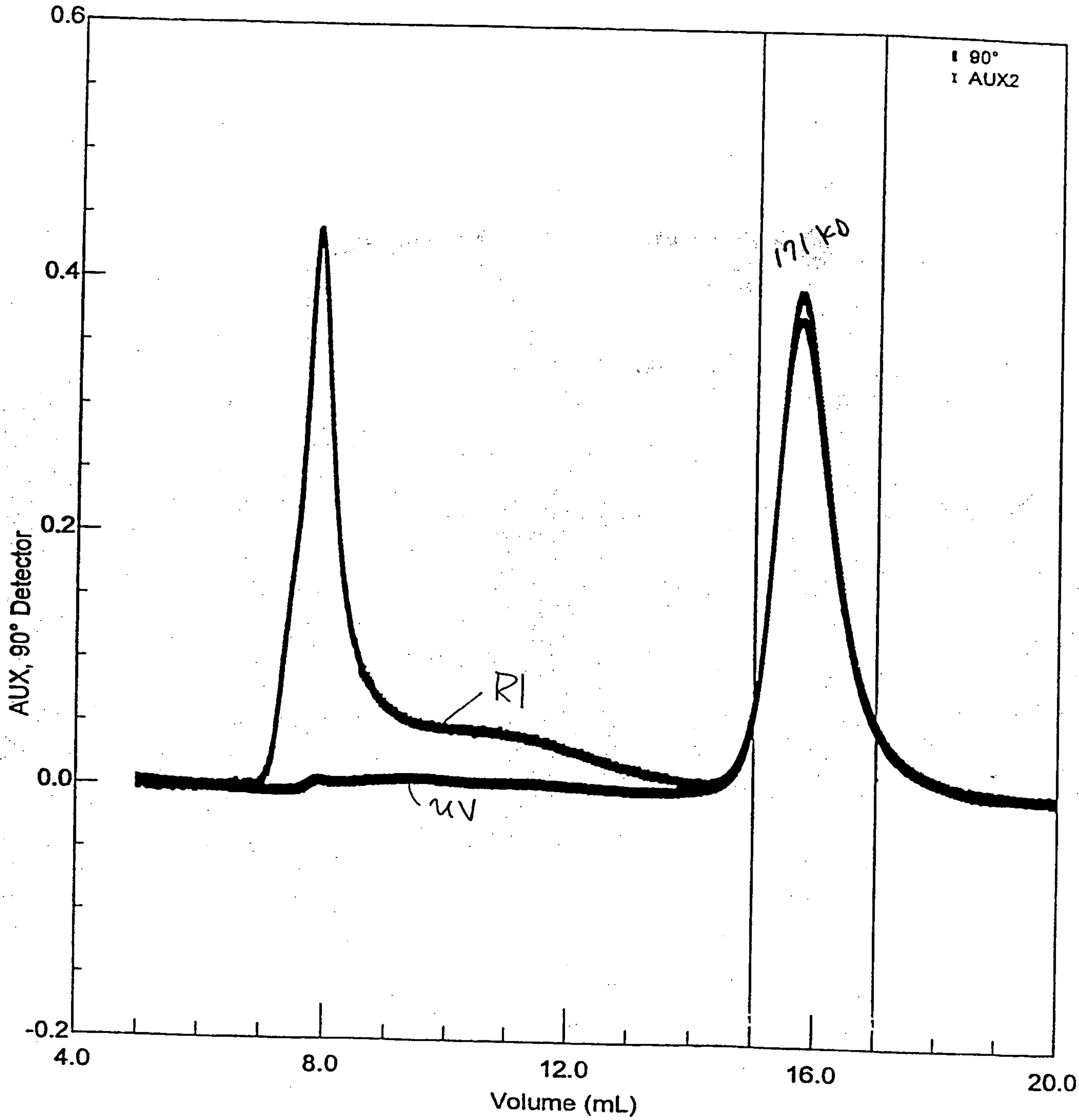
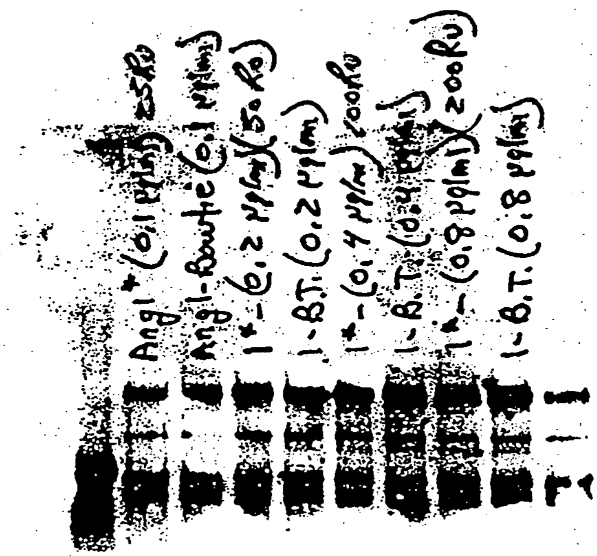

0.6
0.4
0.2
0.0
-0.2
AUX, 90° Detector
4.0
8.0
12.0
16.0
20.0
Volume (mL)
90°
AUX2
171 kD
RI
UV

Figure 9



1 2 3 4 5 6 7 8 9 10

Figure 10

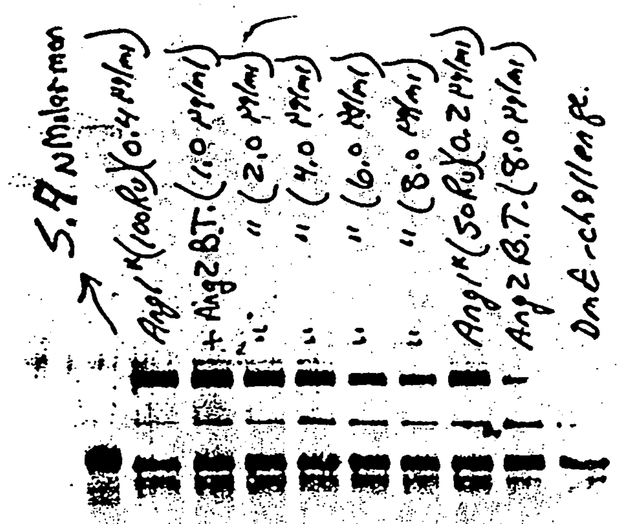
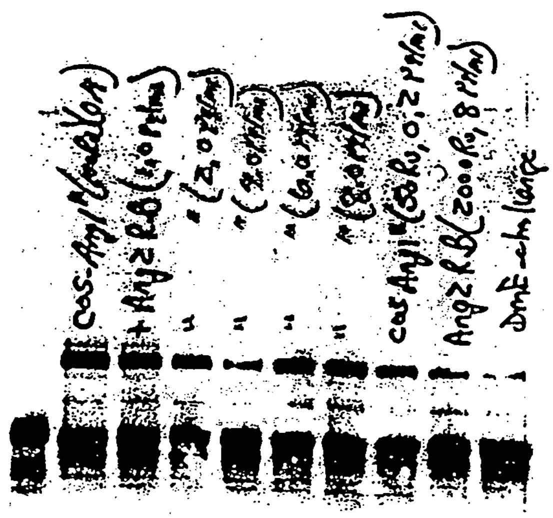


Figure 11



1 2 3 4 5 6 7 8 9 10

Figure 12

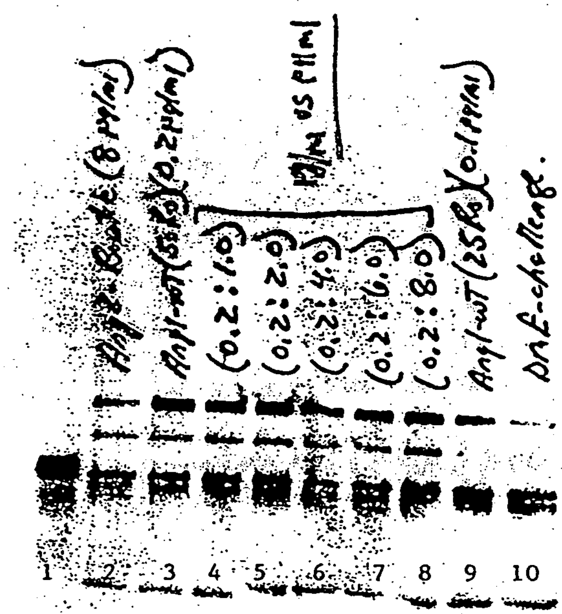
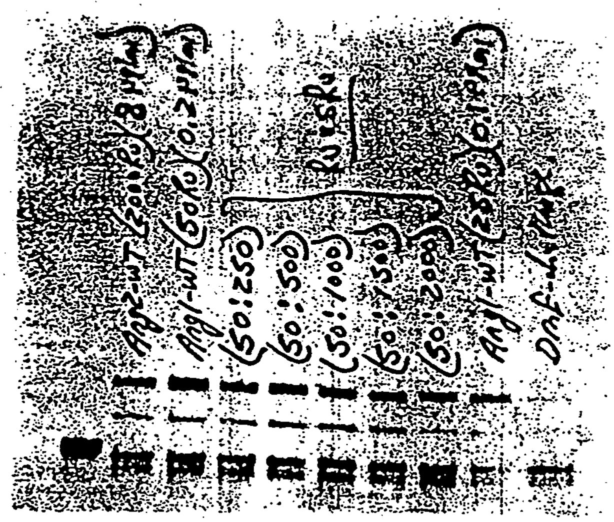


Figure 13



1 2 3 4 5 6 7 8 9 10

Figure 14A

```
         10          20          30          40
          *     *     *     *     *     *     *     *     *
ATG GCT CGG CCT GGG CAG CGT TGG CTC GGC AAG TGG CTT GTG GCG
Met Ala Arg Pro Gly Gln Arg Trp Leu Gly Lys Trp Leu Val Ala>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

    50          60          70          80          90
     *     *     *     *     *     *     *     *     *
ATG GTC GTG TGG GCG CTG TGC CGG CTC GCC ACA CCG CTG GCC AAG
Met Val Val Trp Ala Leu Cys Arg Leu Ala Thr Pro Leu Ala Lys>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

        100         110         120         130
          *     *     *     *     *     *     *     *     *
AAC CTG GAG CCC GTA TCC TGG AGC TCC CTC AAC CCC AAG TTC CTG
Asn Leu Glu Pro Val Ser Trp Ser Ser Leu Asn Pro Lys Phe Leu>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

   140         150         160         170         180
     *     *     *     *     *     *     *     *     *
AGT GGG AAG GGC TTG GTG ATC TAT CCG AAA ATT GGA GAC AAG CTG
Ser Gly Lys Gly Leu Val Ile Tyr Pro Lys Ile Gly Asp Lys Leu>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

        190         200         210         220
          *     *     *     *     *     *     *     *     *
GAC ATC ATC TGC CCC CGA GCA GAA GCA GGG CGG CCC TAT GAG TAC
Asp Ile Ile Cys Pro Arg Ala Glu Ala Gly Arg Pro Tyr Glu Tyr>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

   230         240         250         260         270
     *     *     *     *     *     *     *     *     *
TAC AAG CTG TAC CTG GTG CGG CCT GAG CAG GCA GCT GCC TGT AGC
Tyr Lys Leu Tyr Leu Val Arg Pro Glu Gln Ala Ala Ala Cys Ser>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

        280         290         300         310
          *     *     *     *     *     *     *     *     *
ACA GTT CTC GAC CCC AAC GTG TTG GTC ACC TGC AAT AGG CCA GAG
Thr Val Leu Asp Pro Asn Val Leu Val Thr Cys Asn Arg Pro Glu>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

   320         330         340         350         360
     *     *     *     *     *     *     *     *     *
CAG GAA ATA CGC TTT ACC ATC AAG TTC CAG GAG TTC AGC CCC AAC
Gln Glu Ile Arg Phe Thr Ile Lys Phe Gln Glu Phe Ser Pro Asn>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

        370         380         390         400
          *     *     *     *     *     *     *     *     *
TAC ATG GGC CTG GAG TTC AAG AAG CAC CAT GAT TAC TAC ATT ACC
Tyr Met Gly Leu Glu Phe Lys Lys His His Asp Tyr Tyr Ile Thr>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>
```

Figure 14B

```
  410        420         430        440        450
   *    *    *    *    *    *    *    *    *
TCA ACA TCC AAT GGA AGC CTG GAG GGG CTG GAA AAC CGG GAG GGC
Ser Thr Ser Asn Gly Ser Leu Glu Gly Leu Glu Asn Arg Glu Gly>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

        460        470        480        490
   *    *    *    *    *    *    *    *    *
GGT GTG TGC CGC ACA CGC ACC ATG AAG ATC ATC ATG AAG GTT GGG
Gly Val Cys Arg Thr Arg Thr Met Lys Ile Ile Met Lys Val Gly>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

  500        510         520        530        540
   *    *    *    *    *    *    *    *    *
CAA GAT CCC AAT GCT GTG ACG CCT GAG CAG CTG ACT ACC AGC AGG
Gln Asp Pro Asn Ala Val Thr Pro Glu Gln Leu Thr Thr Ser Arg>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

        550        560        570        580
   *    *    *    *    *    *    *    *    *
CCC AGC AAG GAG GCA GAC AAC ACT GTC AAG ATG GCC ACA CAG GCC
Pro Ser Lys Glu Ala Asp Asn Thr Val Lys Met Ala Thr Gln Ala>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

  590        600         610        620        630
   *    *    *    *    *    *    *    *    *
CCT GGT AGT CGG GGC TCC CTG GGT GAC TCT GAT GGC AAG CAT GAG
Pro Gly Ser Arg Gly Ser Leu Gly Asp Ser Asp Gly Lys His Glu>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

        640        650        660        670
   *    *    *    *    *    *    *    *    *
ACT GTG AAC CAG GAA GAG AAG AGT GGC CCA GGT GCA AGT GGG GGC
Thr Val Asn Gln Glu Glu Lys Ser Gly Pro Gly Ala Ser Gly Gly>
___a___a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a___a___>

  680        690         700        710        720
   *    *    *    *    *    *    *    *    *
AGC AGC GGG GAC CCT GAT GGC TTC TTC AAC TCC AAG GGC CCG GGT
Ser Ser Gly Asp Pro Asp Gly Phe Phe Asn Ser Lys>
___ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)____>
                                                Gly Pro Gly>
                                                ___b___b___>

        730        740        750        760
   *    *    *    *    *    *    *    *    *
AAG AAC CTG GAG CCC GTA TCC TGG AGC TCC CTC AAC CCC AAG TTC
Lys Asn Leu Glu Pro Val Ser Trp Ser Ser Leu Asn Pro Lys Phe>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

  770        780         790        800        810
   *    *    *    *    *    *    *    *    *
CTG AGT GGG AAG GGC TTG GTG ATC TAT CCG AAA ATT GGA GAC AAG
Leu Ser Gly Lys Gly Leu Val Ile Tyr Pro Lys Ile Gly Asp Lys>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>
```

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Figure 14C

```
        820         830         840         850
     *     *     *     *     *     *     *     *     *
CTG GAC ATC ATC TGC CCC CGA GCA GAA GCA GGG CGG CCC TAT GAG
Leu Asp Ile Ile Cys Pro Arg Ala Glu Ala Gly Arg Pro Tyr Glu>

____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

  860         870         880         890         900
     *     *     *     *     *     *     *     *     *
TAC TAC AAG CTG TAC CTG GTG CGG CCT GAG CAG GCA GCT GCC TGT
Tyr Tyr Lys Leu Tyr Leu Val Arg Pro Glu Gln Ala Ala Ala Cys>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

        910         920         930         940
     *     *     *     *     *     *     *     *     *
AGC ACA GTT CTC GAC CCC AAC GTG TTG GTC ACC TGC AAT AGG CCA
Ser Thr Val Leu Asp Pro Asn Val Leu Val Thr Cys Asn Arg Pro>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

  950         960         970         980         990
     *     *     *     *     *     *     *     *     *
GAG CAG GAA ATA CGC TTT ACC ATC AAG TTC CAG GAG TTC AGC CCC
Glu Gln Glu Ile Arg Phe Thr Ile Lys Phe Gln Glu Phe Ser Pro>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

       1000        1010        1020        1030
     *     *     *     *     *     *     *     *     *
AAC TAC ATG GGC CTG GAG TTC AAG AAG CAC CAT GAT TAC TAC ATT
Asn Tyr Met Gly Leu Glu Phe Lys Lys His His Asp Tyr Tyr Ile>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

 1040        1050        1060        1070        1080
     *     *     *     *     *     *     *     *     *
ACC TCA ACA TCC AAT GGA AGC CTG GAG GGG CTG GAA AAC CGG GAG
Thr Ser Thr Ser Asn Gly Ser Leu Glu Gly Leu Glu Asn Arg Glu>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

       1090        1100        1110        1120
     *     *     *     *     *     *     *     *     *
GGC GGT GTG TGC CGC ACA CGC ACC ATG AAG ATC ATC ATG AAG GTT
Gly Gly Val Cys Arg Thr Arg Thr Met Lys Ile Ile Met Lys Val>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

 1130        1140        1150        1160        1170
     *     *     *     *     *     *     *     *     *
GGG CAA GAT CCC AAT GCT GTG ACG CCT GAG CAG CTG ACT ACC AGC
Gly Gln Asp Pro Asn Ala Val Thr Pro Glu Gln Leu Thr Thr Ser>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

       1180        1190        1200        1210
     *     *     *     *     *     *     *     *     *
AGG CCC AGC AAG GAG GCA GAC AAC ACT GTC AAG ATG GCC ACA CAG
Arg Pro Ser Lys Glu Ala Asp Asn Thr Val Lys Met Ala Thr Gln>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

 1220        1230        1240        1250        1260
     *     *     *     *     *     *     *     *     *
GCC CCT GGT AGT CGG GGC TCC CTG GGT GAC TCT GAT GGC AAG CAT
Ala Pro Gly Ser Arg Gly Ser Leu Gly Asp Ser Asp Gly Lys His>
    c    c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c    >
```

Figure 14D

```
        1270         1280         1290         1300
         *           *       *        *          *       *       *       *       *
GAG ACT GTG AAC CAG GAA GAG AAG AGT GGC CCA GGT GCA AGT GGG
Glu Thr Val Asn Gln Glu Glu Lys Ser Gly Pro Gly Ala Ser Gly>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

  1310         1320         1330         1340         1350
     *        *        *        *        *        *        *        *        *
GGC AGC AGC GGG GAC CCT GAT GGC TTC TTC AAC TCC AAA GGC CCG
Gly Ser Ser Gly Asp Pro Asp Gly Phe Phe Asn Ser Lys>
___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___>
                                                    Gly Pro>
                                                    ___d___>

        1360         1370         1380         1390
     *        *        *        *        *        *        *        *        *
GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC
Gly>
___>
    Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys>
    ___e___e___e___e__HUMAN IGG1 FC TAG____e___e___e___e___>

 1400         1410         1420         1430         1440
     *        *        *        *        *        *        *        *        *
CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

        1450         1460         1470         1480
     *        *        *        *        *        *        *        *        *
CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

  1490         1500         1510         1520         1530
     *        *        *        *        *        *        *        *        *
ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG
Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

        1540         1550         1560         1570
     *        *        *        *        *        *        *        *        *
TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

  1580         1590         1600         1610         1620
     *        *        *        *        *        *        *        *        *
AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

        1630         1640         1650         1660
     *        *        *        *        *        *        *        *        *
GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC
Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>
```

Figure 14E

```
     1670          1680           1690          1700          1710
       *       *      *       *       *       *       *       *       *
AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1720          1730          1740          1750
       *       *      *       *       *       *       *       *       *
ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC
Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

     1760          1770           1780          1790          1800
       *       *      *       *       *       *       *       *       *
ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC
Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1810          1820          1830          1840
       *       *      *       *       *       *       *       *       *
CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

     1850          1860           1870          1880          1890
       *       *      *       *       *       *       *       *       *
GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1900          1910          1920          1930
       *       *      *       *       *       *       *       *       *
CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

     1940          1950           1960          1970          1980
       *       *      *       *       *       *       *       *       *
CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1990          2000          2010          2020
       *       *      *       *       *       *       *       *       *
TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

     2030          2040           2050
       *       *      *       *       *
AGC CTC TCC CTG TCT CCG GGT AAA TGA
Ser Leu Ser Leu Ser Pro Gly Lys ***>
___e____HUMAN IGG1 FC TAG__e___e___>
```

Figure 15A

```
           10         20         30         40
 *     *     *     *     *     *     *     *     *
ATG GCC ATG GCC CGG TCC AGG AGG GAC TCT GTG TGG AAG TAC TGT
Met Ala Met Ala Arg Ser Arg Arg Asp Ser Val Trp Lys Tyr Cys>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

    50         60         70         80         90
 *     *     *     *     *     *     *     *     *
TGG GGA CTT TTG ATG GTT TTG TGC AGA ACT GCG ATC TCC AGA TCG
Trp Gly Leu Leu Met Val Leu Cys Arg Thr Ala Ile Ser Arg Ser>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

          100        110        120        130
 *     *     *     *     *     *     *     *     *
ATA GTT TTA GAG CCT ATC TAC TGG AAT TCC TCG AAC TCC AAA TTT
Ile Val Leu Glu Pro Ile Tyr Trp Asn Ser Ser Asn Ser Lys Phe>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

   140        150        160        170        180
 *     *     *     *     *     *     *     *     *
CTA CCC GGA CAA GGC CTG GTA CTA TAC CCA CAG ATA GGA GAC AAA
Leu Pro Gly Gln Gly Leu Val Leu Tyr Pro Gln Ile Gly Asp Lys>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

          190        200        210        220
 *     *     *     *     *     *     *     *     *
TTG GAT ATT ATT TGC CCC AAA GTG GAC TCT AAA ACT GTT GGC CAG
Leu Asp Ile Ile Cys Pro Lys Val Asp Ser Lys Thr Val Gly Gln>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

   230        240        250        260        270
 *     *     *     *     *     *     *     *     *
TAT GAA TAT TAT AAA GTT TAT ATG GTT GAT AAA GAC CAA GCA GAC
Tyr Glu Tyr Tyr Lys Val Tyr Met Val Asp Lys Asp Gln Ala Asp>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

          280        290        300        310
 *     *     *     *     *     *     *     *     *
AGA TGC ACA ATT AAG AAG GAG AAT ACC CCG CTG CTC AAC TGT GCC
Arg Cys Thr Ile Lys Lys Glu Asn Thr Pro Leu Leu Asn Cys Ala>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

   320        330        340        350        360
 *     *     *     *     *     *     *     *     *
AGA CCA GAC CAA GAT GTG AAA TTC ACC ATC AAG TTT CAA GAA TTC
Arg Pro Asp Gln Asp Val Lys Phe Thr Ile Lys Phe Gln Glu Phe>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

          370        380        390        400
 *     *     *     *     *     *     *     *     *
AGC CCT AAC CTC TGG GGT CTA GAA TTT CAG AAG AAC AAA GAT TAC
Ser Pro Asn Leu Trp Gly Leu Glu Phe Gln Lys Asn Lys Asp Tyr>
___a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>
```

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Figure 15B

```
    410          420          430          440          450
     *     *      *     *      *     *      *     *      *
TAC ATT ATA TCT ACA TCA AAT GGG TCT TTG GAG GGC CTG GAT AAC
Tyr Ile Ile Ser Thr Ser Asn Gly Ser Leu Glu Gly Leu Asp Asn>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

           460          470          480          490
     *     *      *     *      *     *      *     *      *
CAG GAG GGA GGG GTG TGC CAG ACA AGA GCC ATG AAG ATC CTC ATG
Gln Glu Gly Gly Val Cys Gln Thr Arg Ala Met Lys Ile Leu Met>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

    500          510          520          530          540
     *     *      *     *      *     *      *     *      *
AAA GTT GGA CAA GAT GCA AGT TCT GCT GGA TCA GCC AGG AAT CAC
Lys Val Gly Gln Asp Ala Ser Ser Ala Gly Ser Ala Arg Asn His>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

           550          560          570          580
     *     *      *     *      *     *      *     *      *
GGT CCA ACA AGA CGT CCA GAG CTA GAA GCT GGT ACA AAT GGG AGA
Gly Pro Thr Arg Arg Pro Glu Leu Glu Ala Gly Thr Asn Gly Arg>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

    590          600          610          620          630
     *     *      *     *      *     *      *     *      *
AGT TCA ACA ACA AGT CCC TTT GTG AAG CCA AAT CCA GGT TCT AGC
Ser Ser Thr Thr Ser Pro Phe Val Lys Pro Asn Pro Gly Ser Ser>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

           640          650          660          670
     *     *      *     *      *     *      *     *      *
ACC GAT GGC AAC AGC GCG GGG CAT TCC GGG AAC AAT CTC CTG GGG
Thr Asp Gly Asn Ser Ala Gly His Ser Gly Asn Asn Leu Leu Gly>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

    680          690          700          710          720
     *     *      *     *      *     *      *     *      *
GGC CCG GGA ATA GTT TTA GAG CCT ATC TAC TGG AAT TCC TCG AAC
Gly Pro Gly>
____b____b____>
            Ile Val Leu Glu Pro Ile Tyr Trp Asn Ser Ser Asn>
            ____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNA_____>

           730          740          750          760
     *     *      *     *      *     *      *     *      *
TCC AAA TTT CTA CCC GGA CAA GGC CTG GTA CTA TAC CCA CAG ATA
Ser Lys Phe Leu Pro Gly Gln Gly Leu Val Leu Tyr Pro Gln Ile>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e____>

    770          780          790          800          810
     *     *      *     *      *     *      *     *      *
GGA GAC AAA TTG GAT ATT ATT TGC CCC AAA GTG GAC TCT AAA ACT
Gly Asp Lys Leu Asp Ile Ile Cys Pro Lys Val Asp Ser Lys Thr>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e____>
```

Figure 15C

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          820                 830                 840                 850
     *     *     *     *     *     *     *     *     *     *
GTT GGC CAG TAT GAA TAT TAT AAA GTT TAT ATG GTT GAT AAA GAC
Val Gly Gln Tyr Glu Tyr Tyr Lys Val Tyr Met Val Asp Lys Asp>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

    860                 870                 880                 890                 900
     *     *     *     *     *     *     *     *     *
CAA GCA GAC AGA TGC ACA ATT AAG AAG GAG AAT ACC CCG CTG CTC
Gln Ala Asp Arg Cys Thr Ile Lys Lys Glu Asn Thr Pro Leu Leu>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

          910                 920                 930                 940
     *     *     *     *     *     *     *     *     *
AAC TGT GCC AGA CCA GAC CAA GAT GTG AAA TTC ACC ATC AAG TTT
Asn Cys Ala Arg Pro Asp Gln Asp Val Lys Phe Thr Ile Lys Phe>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

    950                 960                 970                 980                 990
     *     *     *     *     *     *     *     *     *
CAA GAA TTC AGC CCT AAC CTC TGG GGT CTA GAA TTT CAG AAG AAC
Gln Glu Phe Ser Pro Asn Leu Trp Gly Leu Glu Phe Gln Lys Asn>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

          1000                1010                1020                1030
     *     *     *     *     *     *     *     *     *
AAA GAT TAC TAC ATT ATA TCT ACA TCA AAT GGG TCT TTG GAG GGC
Lys Asp Tyr Tyr Ile Ile Ser Thr Ser Asn Gly Ser Leu Glu Gly>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

    1040                1050                1060                1070                1080
     *     *     *     *     *     *     *     *     *
CTG GAT AAC CAG GAG GGA GGG GTG TGC CAG ACA AGA GCC ATG AAG
Leu Asp Asn Gln Glu Gly Gly Val Cys Gln Thr Arg Ala Met Lys>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

          1090                1100                1110                1120
     *     *     *     *     *     *     *     *     *
ATC CTC ATG AAA GTT GGA CAA GAT GCA AGT TCT GCT GGA TCA GCC
Ile Leu Met Lys Val Gly Gln Asp Ala Ser Ser Ala Gly Ser Ala>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

    1130                1140                1150                1160                1170
     *     *     *     *     *     *     *     *     *
AGG AAT CAC GGT CCA ACA AGA CGC CCA GAG CTA GAA GCT GGT ACA
Arg Asn His Gly Pro Thr Arg Arg Pro Glu Leu Glu Ala Gly Thr>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

          1180                1190                1200                1210
     *     *     *     *     *     *     *     *     *
AAT GGG AGA AGT TCA ACA ACA AGT CCC TTT GTG AAG CCA AAT CCA
Asn Gly Arg Ser Ser Thr Thr Ser Pro Phe Val Lys Pro Asn Pro>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

    1220                1230                1240                1250                1260
     *     *     *     *     *     *     *     *     *
GGT TCT AGC ACC GAT GGC AAC AGC GCG GGG CAT TCC GGG AAC AAT
Gly Ser Ser Thr Asp Gly Asn Ser Ala Gly His Ser Gly Asn Asn>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>
```
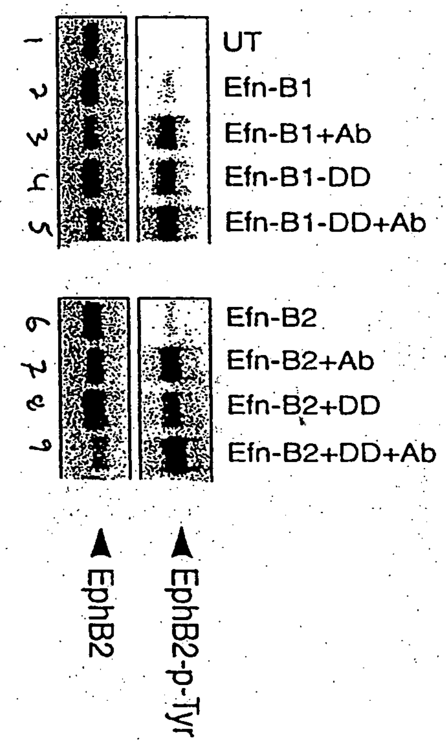

Figure 15D

```
          1270          1280          1290          1300
             *      *      *      *      *      *      *      *      *
CTC CTG GGG G GC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC
                        Glu Pro Lys Ser Cys Asp Lys Thr His>
                        ___c____HUMAN IGG1 FC TAG__c___c___>
             Gly Pro Gly>
             _d__d___d___>
Leu Leu Gly Xxx>
___e___e___e_>

   1310          1320          1330          1340          1350
      *      *      *      *      *      *      *      *      *
ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA
Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1360          1370          1380          1390
      *      *      *      *      *      *      *      *      *
GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

   1400          1410          1420          1430          1440
      *      *      *      *      *      *      *      *      *
CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA
Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1450          1460          1470          1480
      *      *      *      *      *      *      *      *      *
GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG
Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

   1490          1500          1510          1520          1530
      *      *      *      *      *      *      *      *      *
CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG
His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1540          1550          1560          1570
      *      *      *      *      *      *      *      *      *
TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

   1580          1590          1600          1610          1620
      *      *      *      *      *      *      *      *      *
AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA
Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1630          1640          1650          1660
      *      *      *      *      *      *      *      *      *
GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA
Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>
```

Figure 15E

```
 1670          1680           1690          1700          1710
    *       *     *      *      *      *      *      *      *
GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr>
____c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

        1720          1730          1740          1750
    *      *      *      *      *      *      *      *      *
AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC
Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro>
____c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

 1760          1770          1780          1790          1800
    *      *      *      *      *      *      *      *      *
AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC
Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn>
____c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

        1810          1820          1830          1840
    *      *      *      *      *      *      *      *      *
AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe>
____c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

 1850          1860          1870          1880          1890
    *      *      *      *      *      *      *      *      *
TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG
Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln>
____c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

        1900          1910          1920          1930
    *      *      *      *      *      *      *      *      *
GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC
Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn>
____c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

 1940          1950          1960          1970
    *      *      *      *      *      *      *      *
CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
____c___c___c___c__HUMAN IGG1 FC TAG____c___c___c___c___>
```

Figure 16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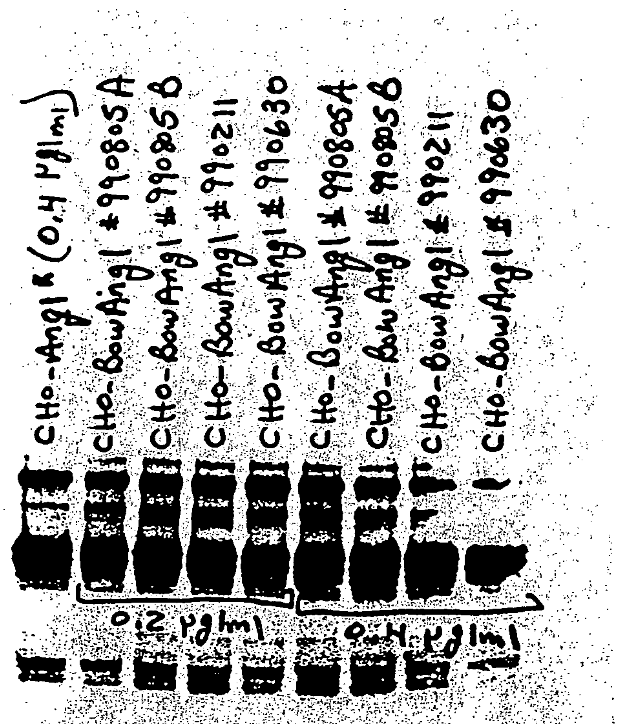
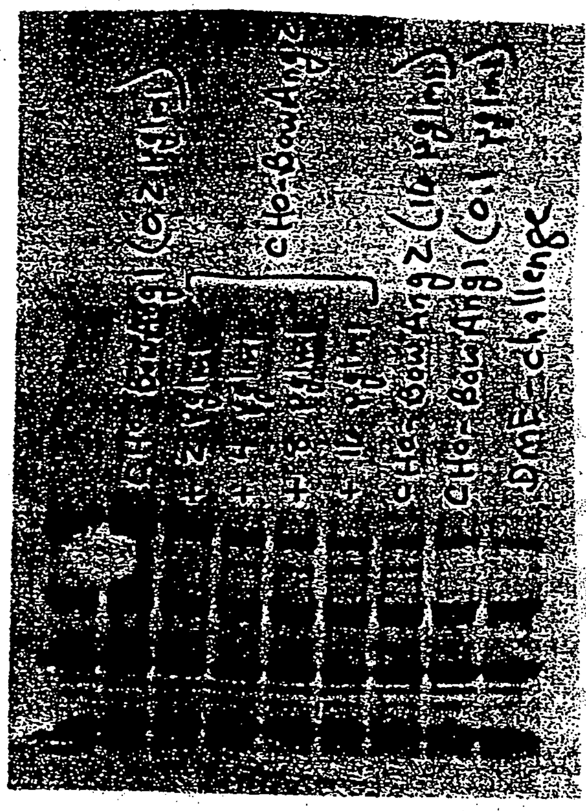
Efn-B1
Efn-B1+Ab
Efn-B1-DD
Efn-B1-DD+Ab
Efn-B2
Efn-B2+Ab
Efn-B2+DD
Efn-B2+DD+Ab
EphB2
EphB2-p-Tyr

Figure 17



1 2 3 4 5 6 7 8 9

Figure 18



1 2 3 4 5 6 7 8 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/30900

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/62 C12N5/10 C12N1/21 C07K14/515
C07K14/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37621 A (MORPHOSYS PROTEINOPTIMIERUNG ;PACK PETER (DE); HOESS ADOLF (DE)) 28 November 1996 (1996-11-28) abstract page 1, line 12 - line 15 page 2, line 4 - line 9 page 14, line 6 - line 11 page 16, line 29 - line 34 figure 1A	1-5, 12-22
Y		8-11
A		6,7

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/30900

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 03569 A (SANGSTAT MEDICAL CORP) 5 March 1992 (1992-03-05) abstract page 1 -page 3 page 19, line 14 - line 23	23-26, 29,32-41
Y		30
A		27,28
Y	WO 95 27060 A (REGENERON PHARMA) 12 October 1995 (1995-10-12) cited in the application abstract page 23, line 21 -page 24, line 8 claims 4,8	8-11,30
A	EP 0 816 510 A (TORAY RESEARCH CENTER INC ;TORAY INDUSTRIES (JP)) 7 January 1998 (1998-01-07) abstract	1-22
A	DAVIS S. ET AL.: "Isolation of Angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning" CELL, vol. 87, 27 December 1996 (1996-12-27), pages 1161-1169, XP002138354 cited in the application the whole document	6,7,27, 28
A	MAISONPIERRE P.C. ET AL.: "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis." SCIENCE, vol. 277, 4 July 1997 (1997-07-04), pages 55-60, XP002138355 cited in the application the whole document	6,7,27, 28
A	TOURNAY C. ET AL.: "Uptake of recombinant myeloperoxidase, free or fused to Fc-gamma, by macrophages enhances killing activity towards micro-organisms" DNA CELL BIOLOGY, vol. 15, no. 8, 1996, pages 617-624, XP000907279 abstract page 618	21,40

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/US 99/30900

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0827544 A	11-03-1998
		JP 11508126 T	21-07-1999
WO 9203569 A	05-03-1992	CA 2090105 A	01-03-1992
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(54) Title: METHOD OF ENHANCING THE BIOLOGICAL ACTIVITY OF LIGANDS

(57) Abstract

Novel fusion polypeptide ligands that bind Eph family receptors or the Tie–2 receptor are identified, and methods for making the fusion polypeptide ligands in biologically active form are described. Nucleic acids encoding these novel fusion polypeptide ligands enable production of the fusion polypeptide ligands. The method of making the nucleic acids and the fusion polypeptide ligands is broadly applicable to the production of polypeptide ligands in general, resulting in improved affinity and/or increased activity of the ligand when compared to its native form.

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METHOD OF ENHANCING THE BIOLOGICAL ACTIVITY OF LIGANDS

This application claims priority of U.S. Application No. 60/113,387, filed December 23, 1998. Throughout this application, various publications are cited. The disclosures of each and all of those publications are hereby incorporated by reference in their entireties into this application.

INTRODUCTION

The present invention provides for novel methods for producing novel fusion polypeptide ligands that have enhanced biological activity as compared to the polypeptide ligands in their native form. The invention also provides for nucleic acids useful for producing biologically active fusion polypeptide ligands, and the fusion polypeptide ligands themselves.

BACKGROUND OF THE INVENTION

The ability of polypeptide ligands to bind cells and thereby elicit a phenotypic response such as cell growth, survival or differentiation is often mediated through transmembrane tyrosine kinases. The extracellular portion of each receptor tyrosine kinase (RTK) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic. Binding of a ligand to the extracellular domain results in signal transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates (Mohammadi, et al.,1990, Mol. Cell. Biol., 11: 5068-5078; Fantl, et al., 1992, Cell, 69:413-413).

RTKs appear to undergo dimerization or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci.

13:443-447; Ullrich and Schlessinger, 1990, Cell, 61:203-212; Schlessinger and Ullrich, 1992, Neuron 9:383-391); molecular interactions between dimerizing cytoplasmic domains lead to activation of kinase function. In some instances, such as the growth factor platelet derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al. , 1988, Science, 240: 1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of EGF, the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem., 259:14631-14636).

Throughout the history of the biotechnology industry, many novel genes and associated proteins have been identified by virtue of their sequence homology with known genes. Many such proteins are purported to be receptors, but since their cognate ligands have not been identified, they are referred to as orphan receptors. The screening of many of these orphan receptors often leads to the identification of ligands that are capable of binding to the receptor, although the binding is often not associated with activation of any intracellular kinases or any other phenotypic change. Such was the case for members of the Eph receptor family. For sake of clarity, applicants incorporate by reference herein a letter cited as Eph Nomenclature Committee, 1997, published in Cell vol. 90: 403-403 (1997) which sets forth a nomenclature for the Eph Receptor and Eph Ligand Families.

Little, if any, biological activity had been observed in response to binding of a ligand to an Eph family receptor prior to the discovery as set forth in U.S. Patent No. 5,747,033 issued May 5, 1998. That patent describes the concept of "clustering" whereby the soluble domains of ligands were combined to create multimers capable of activating the cognate receptors. Applicants have now extended the concept of clustering to additional ligands outside the Eph family, for example, the Tie-2 receptor ligands known as the angiopoietins, and have also discovered that this method for production of homogeneous forms of clustered ligands is broadly applicable to improve

the affinity and/or increase the activity of a ligand as compared to the native form of the ligand.

Angiopoietin-1 (Ang) is one of two known ligands for the Tie-2 receptor and has been shown to be an agonist for Tie-2 (Davis, et al, 1996, Cell 87:1161-1169), whereas the second known ligand, angiopoietin-2, has been shown to be a naturally occurring antagonist of the Tie-2 receptor (Maisonpierre, et al., 1997, Science 277:55-60). Ang1* is a mutant form of angiopoietin-1 that comprises the N-terminal domain of angiopoietin-2 fused to the coiled-coil domain and the fibrinogen domain of angiopoietin-1 and that has a Cys to Ser mutation at amino acid 245. Ang1* has been shown to be a potent agonist for the Tie-2 receptor.

Experiments with mutants of angiopoietin-1 and angiopoietin-2 have demonstrated that the fibrinogen domains (FD) are the receptor-binding domains, and that dimerized versions of, for example Ang-1-FD-Fc, (i.e., the fibrinogen domain of Ang-1 fused to an Fc domain), can bind to the Tie-2 receptor with much higher affinity than monomeric Ang-1-FD (dimerization occurs due to the interaction between the Fc components of adjacent molecules). However, Ang-1-FD-Fc is not able to induce phosphorylation (activate) the Tie-2 receptor on endothelial cells unless it is further clustered with goat anti-human Fc antibodies (Jackson Immunoresearch). For this reason, mutant versions of Ang-1-FD and Ang-2-FD (i.e., the fibrinogen domain of Ang-2) were designed that were intrinsically more highly clustered.

SUMMARY OF THE INVENTION

The present invention provides for novel, biologically active, soluble forms of polypeptide ligands that bind to receptors on cells. Such polypeptide ligands are useful in promoting a differential function and/or influencing the phenotype, such as growth and/or proliferation, of receptor-bearing

cells. The invention also provides for nucleic acids encoding such polypeptide ligands, and both prokaryotic and eukaryotic expression systems for producing such polypeptide ligands. According to the invention, soluble forms of the polypeptide ligands described herein may be used to promote biological responses in receptor-expressing cells. In particular, a general method is described herein which produces fusion polypeptide ligands that may then be clustered, which functions to make otherwise inactive soluble polypeptide ligands biologically active, or which enhances the biological activity of polypeptide ligands that, absent such clustering, would have lower levels of biological activity. This method may be used to cluster a plurality of (more than one) receptor binding domains from any ligand which has improved affinity and/or increased activity (i.e. signaling ability) when clustered as compared to the native form of the ligand.

DESCRIPTION OF THE FIGURES

Figure 1A-1E - Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-FD-Fc.

Figure 2A-2E - Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-FD-Fc.

Figure 3A-3E - Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-Fc-FD.

Figure 4A-4E - Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-Fc-FD.

Figure 5 - Molecular Weight Analysis of Ang-1-FD-Fc-FD protein. SDS PAGE analyses showing a band running at about 210kD under non-reducing conditions (lane 3) and a band running at about 85kD under reducing conditions (lane 7).

Figure 6 - Light scatter analysis to confirm the molecular weight of Ang-1-FD-Fc-FD and to determine whether or not the protein is a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent is monitored with an on line light scattering detector and a refractive index and/or a UV detector. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) is used to calculate the protein concentration. The molecular weight of protein is then calculated from the angular dependence of light scattering. The molecular weight of the dimeric protein appears to be approximately 200kD and presence of a single peak implies that the protein solution is homogenous.

Figure 7 - Molecular Weight Analysis of Ang-2-FD-Fc-FD. SDS PAGE analyses showing a band running at about 200kD under non-reducing conditions (lanes 7 and 8) and a band running at about 88kD under reducing conditions (lanes 3 and 4).

Figure 8 - Light scatter analysis to confirm the molecular weight of Ang-2-FD-Fc-FD and to determine whether or not the protein is a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent is monitored with an on line light scattering detector and a refractive index and/or a UV detector. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) is used to calculate the protein concentration. The molecular weight of protein is then calculated from the angular dependence of light scattering. The molecular weight of the dimeric protein appears to be approximately 171kD and presence of a single peak implies that the

protein solution is homogenous.

Figure 9 - Ang1*-mediated vs. Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. A standard phosphorylation assay revealed that Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926.

Figure 10 - Ability of Ang-2-FD-Fc-FD to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells. In a standard phosphorylation assay, Ang-2-FD-Fc-FD is able to block Ang1* stimulation of the Tie-2 receptor when it is present in at least a 10-15 fold molar excess of Ang1*.

Figure 11 - Ability of angiopoietin-2 to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells. In a standard phosphorylation assay, at a 20 fold molar excess, angiopoietin-2 is not able to reduce the Ang1*-mediated phosphorylation level to 50%. This result, coupled with the results described in Figure 10 implies that Ang-2-FD-Fc-FD is a more potent inhibitor of Ang1*-mediated Tie-2 receptor phosphorylation than angiopoietin-2.

Figure 12 - Ability of Ang-2-FD-Fc-FD to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells. In a standard phosphorylation assay, it is shown that while there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, Ang-2-FD-Fc-FD seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 10.

Figure 13 - Ability of angiopoietin-2 to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells. In a standard phosphorylation assay, it is shown that there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, but, like Ang-2-FD-Fc-FD, angiopoietin-2 seems to be more effective at

blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 11.

Figure 14A-14E - Nucleic acid sequence and deduced amino acid sequence of Ephrin-B1-Ephrin-B1-Fc.

Figure 15A-15E - Nucleic acid sequence and deduced amino acid sequence of Ephrin-B2-Ephrin-B2-Fc.

Figure 16 - Comparison of Ephrin-B1-Fc, Ephrin-B1-Ephrin-B1-Fc, Ephrin-B2-Fc and Ephrin-B2-Ephrin-B2-Fc in standard EphB2 phosphorylation assays. COS cells were serum-starved and then left untreated (UT), lane 1, or were treated with unclustered and clustered Ephrin-B1-Fc (Efn-B1), lanes 2 and 3. COS cells were also treated with unclustered and clustered Ephrin-B1-Ephrin-B1-Fc (Efn-B1 DD), lanes 4 and 5. In addition cells were likewise treated with unclustered and clustered Ephrin-B2-Fc (Efn-B2), lanes 6 and 7 and with unclustered and clustered Ephrin-B2-Ephrin-B2-Fc (Efn-B2 DD), lanes 8 and 9. The extent of EphB2 phosphorylation was assessed by anti-phosphotyrosine western blotting (upper panels) and the relative amounts of EphB2 in each lane was determined by anti-EphB2 western blotting (lower panels).

Figure 17 - Ang1*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were stimulated with 0.4 μg/ml Ang1* or 0.2 μg/ml or 0.4 μg/ml stable CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells.

Figure 18 - Ability of stable CHO clone-derived Ang-2-FD-Fc-FD to block stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were treated with 0.2

μg/ml of the Tie-2 agonist Ang-1-FD-Fc-FD and 2 μg/ml, 4 μg/ml, 8 μg/ml or 16 μg/ml of stable CHO clone-derived Ang-2-FD-Fc-FD. Ang-2-FD-Fc-FD is able to block or stable CHO clone-derived Ang-1-FD-Fc-FD stimulation of the Tie-2 receptor when it is present in at least a 40 fold molar excess of stable CHO clone-derived Ang-1-FD-Fc-FD.

DETAILED DESCRIPTION OF THE INVENTION

As described in greater detail below, applicants have discovered a method for "clustering" polypeptide ligands, which functions to make otherwise inactive soluble polypeptide ligands biologically active, or which enhances the biological activity of polypeptide ligands that, absent such clustering, would have lower levels of biological activity. This method may be used to cluster a plurality of (more than one) receptor binding domains from any ligand which has improved affinity and/or increased activity (i.e. signaling ability) when clustered as compared to the native form of the ligand.

The present invention provides for a nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of the receptor binding domain of a ligand, the first subunit being fused to the N-terminal end of a multimerizing component, said multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of a ligand.

In one embodiment of the invention, the receptor binding domains of the first and second subunits are copies of the receptor binding domain of the same ligand. The first and second subunits may each have one or more than one copy of the receptor binding domain of the ligand. In specific embodiments of the invention, the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2. Alternatively, the

receptor binding domain is from a ligand selected from the group consisting of the EPH family of ligands (i.e., the ephrins).

In another embodiment of the invention, the receptor binding domains of the first subunit are copies of the receptor binding domain of a different ligand from the receptor binding domains of the second subunit. For example, the first subunit may comprise the receptor binding domain of an angiopoietin and the second subunit may comprise the receptor binding domain of vascular endothelial growth factor (VEGF). Alternatively, the first subunit may comprise the receptor binding domain of VEGF and the second subunit may comprise the receptor binding domain an angiopoietin. Still further, the first and second subunits may each have one or more than one copy of the receptor binding domain of their respective ligand.

By "receptor binding domain" what is meant is the minimal portion of the ligand that is necessary to bind its receptor.

In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(ΔC1).

The present invention also provides for fusion polypeptides encoded by the nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are described in European Patent Application of ZymoGenetics, Inc., Publication No. EP 0 721 983 A1 published 17 July 1996

and include S. cerevisiae repressible acid phosphatase (Mizunaga et al., 1988, J. Biochem. (Tokyo) 103:321-326); the S. cerevisiae type 1 killer preprotoxin (Sturley et al., 1986, EMBO J. 5:3381-3390); the S. calsbergensis alpha galactosidase melibiase (Sumner-Smith, et al., 1985, Gene 36:333-340); and the Neurospora crassa ornithine decarboxylase (Digangi, et al., 1987, J. Biol. Chem. 262:7889-7893). Sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); the S. cerevisiae SUC2 gene (Carlson et al., 1983, Mol. Cell. Biol. 3:439-447); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS or CHO cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The fusion polypeptides useful for practicing the present invention may be prepared by expression in a prokaryotic or eukaryotic expression system.

The recombinant gene may be expressed and the polypeptide purified utilizing any number of methods. The gene may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The fusion polypeptides may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The present invention also provides for a nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises more than one copy of the receptor binding domain of a ligand in tandem, and wherein either the N-terminal or the C-terminal receptor binding domain is also fused to a multimerizing component. In one embodiment of the invention, the receptor binding domains are fused contiguously. In another embodiment of the invention, the receptor binding domains are from a ligand that is not a member of the EPH family of ligands (i.e., not an ephrin). In specific embodiments of the invention, the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2. In an alternative embodiment, the receptor binding domain is from vascular endothelial growth factor (VEGF). In another embodiment, the receptor binding domain is from an ephrin.

By "receptor binding domain" what is meant is the minimal portion of the ligand that is necessary to bind its receptor.

In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(ΔC1).

The present invention also provides for fusion polypeptides encoded by the nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are described in European Patent Application of ZymoGenetics, Inc., Publication No. EP 0 721 983 A1 published 17 July 1996 and include S. cerevisiae repressible acid phosphatase (Mizunaga et al., 1988, J. Biochem. (Tokyo) 103:321-326); the S. cerevisiae type 1 killer preprotoxin (Sturley et al., 1986, EMBO J. 5:3381-3390); the S. calsbergensis alpha galactosidase melibiase (Sumner-Smith, et al., 1985, Gene 36:333-340); and the Neurospora crassa ornithine decarboxylase (Digangi, et al., 1987, J. Biol. Chem. 262:7889-7893). Sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); the S. cerevisiae SUC2 gene (Carlson et al., 1983, Mol. Cell. Biol. 3:439-447); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is

operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS or CHO cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The fusion polypeptides useful for practicing the present invention may be prepared by expression in a prokaryotic or eukaryotic expression system. The recombinant gene may be expressed and the polypeptide purified utilizing any number of methods. The gene may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The fusion polypeptides may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The Examples describe the preparation of novel polypeptide ligands that comprise a receptor binding domain of a member of the Eph (Eph transmembrane tyrosine kinase family ligands) family of ligands or of a

member of the angiopoietin family of ligands that can bind the Tie-2 receptor.

For a description of novel Eph family ligands, methods of making and using them, as well as the sequences of EHK-1L, B61 and ELK-L, together with a description of a method of enhancing the biological activity of EPH family ligands by clustering them, applicants refer to U.S. Patent No. 5,747,033 issued on May 5, 1998 which is hereby incorporated by reference in its entirety. Applicants further refer to International Application PCT/US93/10879, published as WO 94/11020 on May 26, 1994; and International Application PCT/US96/17201 published as WO 97/15667 entitled "Biologically Active EPH Family Ligands" each of which is hereby incorporated by reference in its entirety.

As has been previously reported, a family of ligands for the TIE-2 receptor has been discovered and named the Angiopoietins. This family, consisting of TIE-2 ligand 1 (Ang-1); TIE-2 ligand 2 (Ang-2); TIE ligand 3 (Ang-3); and TIE ligand 4 (Ang-4) has been extensively characterized. For a description of the cloning, sequencing and characterization of the angiopoietins, as well as for methods of making and uses thereof, including the production and characterization of modified and chimeric ligands thereof, reference is hereby made to the following publications, each of which is incorporated by reference herein in its entirety: U.S. Patent No. 5,521,073 issued May 28, 1996; U.S. Patent No. 5,643,755 issued July 1, 1997; U.S. Patent No. 5,650,490 issued July 22, 1997; U.S. Patent No. 5,814,464 issued September 29, 1998; U.S. Patent No. 5,879,672 issued March 9, 1999; U.S. Patent No. 5,851,797 issued December 22, 1998; PCT International Application entitled "TIE-2 Ligands Methods of Making and Uses Thereof," published as WO 96/11269 on 18 April 1996 in the name of Regeneron Pharmaceuticals, Inc.; PCT International Application entitled "TIE-2 Ligands Methods of Making and Uses Thereof,"published as WO 96/31598 on 10 October 1996 in the name of Regeneron Pharmaceuticals, Inc.; PCT International Application entitled

"TIE-2 Receptor Ligands (TIE Ligand-3; TIE Ligand-4) And Their Uses," published as WO 97/48804 on 24 December 1997 in the name of Regeneron Pharmaceuticals, Inc; and PCT International Application entitled "Modified TIE-2 Receptor Ligands," published as WO 98/05779 on 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc.

When used herein, fusion polypeptide includes functionally equivalent molecules in which amino acid residues are substituted for residues within the sequence resulting in a silent or conservative change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent or conservative alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are proteins or fragments or derivatives thereof which exhibit the same or similar biological activity and derivatives which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Cells that express the fusion polypeptides of the invention are genetically engineered to produce them by, for example, transfection, transduction, electroporation, or microinjection.

The present invention encompasses the nucleic acid sequences encoding the fusion polypeptides of the invention, as well as sequences that hybridize under stringent conditions to nucleic acid sequences that are complementary to the nucleic acid sequences of the invention. Stringent conditions are set forth in, for example, Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2 ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989). In addition, the present invention encompasses nucleic acid sequences that are different from the nucleic acid sequences of the invention but which nevertheless encode the fusion polypeptides of the invention due to the degeneracy of the genetic code.

In addition, the present invention contemplates use of the fusion polypeptides described herein in tagged forms.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the fusion polypeptides of the invention using appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence encoding the fusion polypeptides of the invention may be regulated by a second nucleic acid sequence so that the fusion polypeptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the fusion polypeptides described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the fusion polypeptide include, but are not limited to the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980,

Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene

control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising Eph fusion polypeptide encoding or angiopoietin fusion polypeptide encoding nucleic acids as described herein, are used to transfect the host and thereby direct expression of such nucleic acid to produce fusion polypeptides which may then be recovered in biologically active form. As used herein, a biologically active form includes a form capable of binding to the relevant receptor and causing a differentiated function and/or influencing the phenotype of the cell expressing the receptor. Such biologically active forms would, for example, induce phosphorylation of the tyrosine kinase domain of the Ehk-1, Elk, or Tie2 receptor, or stimulation of synthesis of cellular DNA.

Expression vectors containing the nucleic acid inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign nucleic acids inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted nucleic acid sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign nucleic acid sequences in the vector. For example, if an efl nucleic acid sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign nucleic acid product expressed by the recombinant. Such assays can be based, for example, on the physical or

functional properties of the nucleic acid product of interest, for example, by binding of a ligand to a receptor or portion thereof which may be tagged with, for example, a detectable antibody or portion thereof or binding to antibodies produced against the protein of interest or a portion thereof.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the ephrin or angiopoietin fusion polypeptide as described herein.

The ephrin fusion polypeptides of the invention may be useful in methods of treating a patient suffering from a neurological disorder comprising treating the patient with an effective amount of the ephrin fusion polypeptide.

For example, the Elk receptor is expressed primarily in brain. Accordingly, it is believed that an Elk binding ephrin fusion polypeptide ligand will support the induction of a differential function and/or influence the phenotype, such as growth and/or survival of neural cells that express this receptor.

The present invention also provides for pharmaceutical compositions comprising the ephrin fusion polypeptide in a suitable pharmacologic carrier. The compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

As our understanding of neurodegenerative disease/neurotrauma becomes clearer, it may become apparent that it would be beneficial to decrease the effect of endogenous Efl-6. Therefore, in areas of nervous system trauma, it may be desirable to provide Efl-6 antagonists, including, but not limited to,

fusion polypeptide forms of Efl-6 which may compete with cell-bound ligand for interaction with Elk receptor. It may be desirable to provide such antagonists locally at the injury site rather than systemically. Use of an Efl-6 antagonist providing implant may be desirable.

Alternatively, certain conditions may benefit from an increase in Efl-6 responsiveness. It may therefore be beneficial to increase the number or binding affinity of Efl-6 in patients suffering from such conditions.

The invention herein further provides for the development of a fusion polypeptide, as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the TIE-2 receptor. Such molecules may be used in a method of treatment of the human or animal body, or in a method of diagnosis.

Because TIE-2 receptor has been identified in association with endothelial cells and, as was previously demonstrated, blocking of agonists of the receptor such as TIE-2 ligand 1 (Ang-1) has been shown to prevent vascularization, applicants expect that TIE-2 agonist fusion polypeptides of the invention may be useful for the induction of vascularization in diseases or disorders where such vascularization is indicated. Such diseases or disorders would include wound healing, ischemia and diabetes. The ligands may be tested in animal models and used therapeutically as described for other agents, such as vascular endothelial growth factor (VEGF), another endothelial cell-specific factor that is angiogenic. Ferrara, et al. U.S. Patent No. 5,332,671 issued July 26, 1994. The Ferrara reference, as well as other studies, describe <u>in vitro</u> and <u>in vivo</u> studies that may be used to demonstrate the effect of an angiogenic factor in enhancing blood flow to ischemic myocardium, enhancing wound healing, and in other therapeutic settings wherein neoangiogenesis is desired. [see Sudo, et al., European Patent Application 0 550 296 A2 published July 7, 1993; Banai, et al. Circulation 89:2183-2189 (1994); Unger, et al. Am. J. Physiol. 266:H1588-H1595

(1994); Lazarous, et al. Circulation 91:145-153 (1995)]. According to the invention, the agonist fusion polypeptides may be used alone or in combination with one or more additional pharmaceutically active compounds such as, for example, VEGF or basic fibroblast growth factor (bFGF).

Conversely, antagonists of the TIE-2 receptor, such as TIE-2 receptorbodies or TIE-2 ligand 2 (Ang-2) as described in Example 9 in International Publication No. WO 96/31598 published 10 October 1996, have been shown to prevent or attenuate vascularization, and are thus expected to be useful in preventing or attenuating, for example, tumor growth. Similarly then, TIE-2 antagonist fusion polypeptides of the invention would also be useful for those purposes. These antagonists may be used alone or in combination with other compositions, such as anti-VEGF antibodies, that have been shown to be useful in treating conditions in which the therapeutic intent is to block angiogenesis.

For example, applicants have determined that TIE-2 ligands are expressed in cells within, or closely associated with, tumors. For example, TIE-2 ligand 2 (Ang-2) appears to be tightly associated with tumor endothelial cells. Accordingly, TIE-2 antagonist fusion polypeptides of the invention may also be useful in preventing or attenuating, for example, tumor growth.

In other embodiments, the TIE-2 agonist fusion polypeptides of the invention described herein may be used as hematopoietic factors. A variety of hematopoietic factors and their receptors are involved in the proliferation and/or differentiation and/or migration of the various cells types contained within blood. Because the TIE-2 receptors are expressed in early hematopoietic cells, the TIE-2 ligands are expected to play a comparable role in the proliferation or differentiation or migration of these cells. Thus, for example, TIE-2 agonist fusion polypeptide compositions may be prepared, assayed, examined in <u>in vitro</u> and <u>in vivo</u> biological systems and

used therapeutically as described in any of the following: Sousa, U.S. Patent No. 4,810,643, Lee, et al., Proc. Natl. Acad. Sci. USA 82:4360-4364 (1985) Wong, et al. Science, 228:810-814 (1985); Yokota, et al. Proc. Natl. Acad. Sci (USA) 81:1070 (1984); Bosselman, et al. WO 9105795 published May 2, 1991 entitled "Stem Cell Factor" and Kirkness, et al. WO 95/19985 published July 27, 1995 entitled "Haemopoietic Maturation Factor". Accordingly, the fusion polypeptides may be used to diagnose or treat conditions in which normal hematopoiesis is suppressed, including, but not limited to anemia, thrombocytopenia, leukopenia and granulocytopenia. In a preferred embodiment, the fusion polypeptides may be used to stimulate differentiation of blood cell precursors in situations where a patient has a disease, such as acquired immune deficiency syndrome (AIDS) which has caused a reduction in normal blood cell levels, or in clinical settings in which enhancement of hematopoietic populations is desired, such as in conjunction with bone marrow transplant, or in the treatment of aplasia or myelosuppression caused by radiation, chemical treatment or chemotherapy.

The fusion polypeptides of the present invention may be used alone, or in combination with another pharmaceutically active agents such as, for example, cytokines, neurotrophins, interleukins, etc. In a preferred embodiment, the fusion polypeptides may be used in conjunction with any of a number of factors which are known to induce stem cell or other hematopoietic precursor proliferation, or factors acting on later cells in the hematopoietic pathway, including, but not limited to, hemopoietic maturation factor, thrombopoietin, stem cell factor, erythropoietin, G-CSF, GM-CSF, etc.

In an alternative embodiment, TIE-2 receptor antagonist fusion polypeptides are used to diagnose or treat patients in which the desired result is inhibition of a hematopoietic pathway, such as for the treatment of myeloproliferative or other proliferative disorders of blood forming organs

such as thrombocythemias, polycythemias and leukemias. In such embodiments, treatment may comprise use of a therapeutically effective amount of the fusion polypeptides as described herein.

Effective doses useful for treating these or other diseases or disorders may be determined using methods known to one skilled in the art [see, for example, Fingl, et al., The Pharmacological Basis of Therapeutics, Goodman and Gilman, eds. Macmillan Publishing Co., New York, pp. 1-46 ((1975)]. Pharmaceutical compositions for use according to the invention include the fusion polypeptides described above in a pharmacologically acceptable liquid, solid or semi-solid carrier, linked to a carrier or targeting molecule (e.g., antibody, hormone, growth factor, etc.) and/or incorporated into liposomes, microcapsules, and controlled release preparation prior to administration *in vivo*. For example, the pharmaceutical composition may comprise a fusion polypeptide in an aqueous solution, such as sterile water, saline, phosphate buffer or dextrose solution. Alternatively, the active agents may be comprised in a solid (e.g. wax) or semi-solid (e.g. gelatinous) formulation that may be implanted into a patient in need of such treatment. The administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an implanted device.

Administration may result in the distribution of the active agent of the invention throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of agent may be desirable. In some situations, an implant containing active agent may be placed in or near the lesioned area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

The present invention also provides for pharmaceutical compositions comprising the fusion polypeptides described herein, in a pharmacologically acceptable vehicle. The compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

EXAMPLES

Angiopoietin ligands:

As described *supra*, experiments with mutants of Ang-1 and Ang-2 have demonstrated that the fibrinogen domains (FD) are the receptor-binding domains, and that dimerized versions (dimerization occurs due to the interaction between the Fc components of adjacent molecules), for example Ang-1-FD-Fc, can bind to the Tie-2 receptor with much higher affinity than monomeric Ang-1-FD. However, Ang-1-FD-Fc is not able to induce phosphorylation (activate) the Tie-2 receptor on endothelial cells unless it is further clustered with goat anti-human Fc antibodies (Jackson Immunoresearch). For this reason, mutant versions of Ang-1-FD and Ang-2-FD were designed that were intrinsically more highly clustered.

Two general types of nucleic acid molecules were constructed. The first type consisted of two tandem copies of Ang-1-FD fused to an Fc tag, thus leading

to a secreted polypeptide molecule that is dimeric with respect to the Fc tag but tetrameric with respect to Ang-1-FD. Similarly, two tandem copies of Ang-2-FD fused to an Fc tag constituted the angiopoietin-2 version of this type of construct. These molecules were designated Ang-1-FD-FD-Fc and Ang-2-FD-FD-Fc, respectively.

In the second type of nucleic acid molecule constructed, two copies of Ang-1-FD were connected by an Fc tag bridging between them, thus creating the structure Ang-1-FD-Fc-FD that is still dimeric with respect to the Fc, as well as tetrameric with respect to Ang-1-FD. An angiopoietin-2 version was also constructed and these two molecules were designated Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD, respectively.

For either type of construct, similar properties were observed: unlike dimeric Ang-1-FD-Fc, which fails to activate Tie-2 in endothelial cells, both Ang-1-FD-FD-Fc and Ang-1-FD-Fc-FD could readily activate Tie-2 in endothelial cells, with a potency comparable to that of the native ligand. Also, like native angiopoietin-2, Ang-2-FD-Fc-FD could antagonize angiopoietin-1 activity with a potency that is comparable to that of native angiopoietin-2, and with much greater potency than the marginally antagonistic activity of the Ang-2-FD-Fc dimer.

<u>Construction of mutant angiopoietin nucleic acid molecules</u>.

All of the following nucleic acid molecules were constructed by standard recombinant DNA techniques (See e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), sequence-verified by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and subcloned into the mammalian expression vector pMT21 (Genetics

Institute, Inc.) with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5′ end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

Example 1: Construction of the Ang-1-FD-FD-Fc, Ang-2-FD-FD-Fc, Ang-1-FD-Fc-FD, and Ang-2-FD-Fc-FD nucleic acid molecules.

Ang-1-FD-FD-Fc: Ang-1-FD-FD-Fc consists of a trypsin signal sequence at its amino terminus to allow for secretion (bases 1-45 of Figure 1A) followed by the angiopoietin-1 fibrinogen domain (FD) (bases 46-690 of Figure 1A-Figure 1B), a short bridging sequence consisting of the amino acids Gly-Pro Ala-Pro (bases 691-702 of Figure 1B), a second angiopoietin-1 FD (bases 703-1750 of Figure 1B-Figure 1D), another bridging sequence consisting of the amino acids Gly-Pro-Gly (bases 1351-1359 of Figure 1D), and the coding sequence for the Fc portion of human IgG1 (bases 1360-2058 of Figure 1D-Figure 1E).

Ang-2-FD-FD-Fc: The Ang-2-FD-FD-Fc nucleic acid molecule was similarly constructed. It consists of a trypsin signal sequence (bases 1-45 of Figure 2A), an angiopoietin-2 FD (bases 46-690 of Figure 2A- Figure 2B), a bridging amino acid sequence Gly-Gly-Pro-Ala-Pro (bases 691-705 of Figure 2B), a second angiopoietin-2 FD (bases 706-1353 of Figure 2B-Figure 2D), another bridging amino acid sequence Gly-Pro-Gly (bases 1354-1362 of Figure 2D), and the coding sequence for the Fc portion of human IgG1 (bases 1363-2061 of Figure 2D-Figure 2E.

Ang-1-FD-Fc-FD: The Ang-1-FD-Fc-FD consists of a trypsin signal sequence (bases 1-45 of Figure 3A), an angiopoietin-1 FD (bases 46-690 of Figure 3A-3B), the bridging amino acid sequence Gly-Pro-Gly (bases 691-699 of Figure

3B), the coding sequence for the Fc portion of human IgG1 (bases 700-1395 of Figure 3B-3D), another bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1396-1419 of Figure 3D), and a second angiopoietin-1 FD (bases 1420-2067 of Figure 3D-Figure 3E).

__Ang-2-FD-Fc-FD:__ The Ang-2-FD-Fc-FD nucleic acid molecule consists of a trypsin signal sequence (bases 1-45 of Figure 4A), an angiopoietin-2 FD domain (bases 46-690 of Figure 4A-Figure 4B), the bridging amino acid sequence Gly-Gly-Pro-Gly (bases 691-702 of Figure 4B), the coding sequence for the Fc portion of human IgG1 (bases 703-1398 of Figure 4B- Figure 4D), the bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1399-1422 of Figure 4D), and a second angiopoietin-2 FD (bases 1423-2067 of Figure 4D-Figure 4E).

__Example 2: Characterization of Ang-1 FD-Fc-FD protein.__

__Molecular Weight Analysis__: The predicted molecular weight for Ang-1-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form (with respect to the Fc) has a predicted weight of 76,349. In addition, there are three predicted N-linked glycosylation sites, approximately 2500 MW/site, that could potentially increase the molecular weight of the monomeric protein to 83,849. Due to the interaction between the Fc components of adjacent molecules, the protein actually exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,698. Subsequent SDS PAGE analyses of COS cell-derived protein described *infra* confirmed these approximate molecular weights, with a band running at about 210kD under non-reducing conditions and a band running at about 85kD under reducing conditions (Figure 5). Light scatter analysis was performed to further confirm the molecular weight and, more importantly, determine whether or not the protein was a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To

determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent was monitored with an on line light scattering detector and a refractive index and/or a UV detector. The light scattering detector is a MiniDawn laser light scattering detector was from Wyatt Technology Corporation (Santa Barbara, CA). This instrument measures static light at three different angles. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) was used to calculate the protein concentration based on either dn/dc (dn = change of refractive index; dc = concentration) or the extinction coefficient of the protein. The molecular weight of protein is then calculated from the angular dependence of light scattering. Figure 6 shows the results of this analysis using COS cell-derived protein. The molecular weight of the dimeric protein appears to be approximately 200kD and presence of a single peak implies that the protein solution is, in fact, homogenous.

<u>Expression Level in COS Cells:</u> COS cell supernatant containing recombinant Ang-1-FD-Fc-FD protein was generated by transiently transfecting COS cells with the Ang1-FD-Fc-FD DNA construct described *supra*. All transfections were performed using standard techniques known in the art. The COS cell supernatant was analyzed using Biacore technology (Pharmacia, Inc.) to quantitate the amount of Ang-1-FD-Fc-FD protein present in the supernatant. This analysis resulted in an RU value of 765, which is equivalent to 0.9mg of recombinant protein/liter of COS cell supernatant. These values represent very high levels of expression.

<u>Purification of COS Supernatants</u>: Because the Ang-1-FD-Fc-FD protein contains an Fc domain, purification is relatively simple and straight forward using standard Protein A column chromatography (Pharmacia, Inc.) followed by standard size exclusion chromatography (Pharmacia, Inc.). In fact, the relative ease of purification of the Ang-1-FD-Fc-FD protein gives it a distinct advantage over the parent protein, angiopoietin-1, from which

it is derived, and the mutant version of angiopoietin-1 called Ang1* that consists of the N-terminal of angiopoietin-2 fused to the coiled-coil domain and fibrinogen domain of angiopoietin-1 and that has a Cys to Ser mutation at amino acid 245. (See PCT International Application entitled "Modified TIE-2 Receptor Ligands," published as WO 98/05779 on 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc., especially Figure 27, which is hereby incorporated by reference).

Both angiopoietin-1 and Ang1* require extensive, expensive and labor-intensive purification schemes that result in relatively poor yields of recombinant protein. The need for cost-effective, simple purification schemes for biologicals intended for clinical use can not be over-emphasized.

The COS cell supernatant was purified as described *supra* and yielded approximately 1 mg of purified Ang-1-FD-Fc-FD protein that was used in the studies described *infra* to further characterize the protein.

<u>N-terminal sequencing of COS cell-derived Ang-1-FD-Fc-FD protein</u>: Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. This was of concern because the mutant molecule, Ang1*, has a history of containing between 10-20% N-terminally truncated species. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys. This sequence can be found at amino acids 16-20 of Figure 3A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 Figure 3A.

<u>Receptor binding analysis of COS cell-derived Ang-1-FD-Fc-FD</u>: Previous studies have determined that the fibrinogen domain (FD) of the angiopoietin molecules is necessary for ligand/receptor interaction. Furthermore, in order for high affinity binding to the Tie-2 receptor to

occur, native angiopoietin-1, angiopoietin-2, and the mutant Ang1* must form at least tetrameric, and possibly higher order, multimers. To determine whether the COS cell-derived Ang-1-FD-Fc-FD protein, which is tetrameric with respect to the FD domain, could bind to Tie-2 with high affinity, standard Biacore analysis was performed. Briefly, Tie-2-Fc receptor body protein, which is a fusion protein comprising the ectodomain of Tie-2 fused to the Fc domain of human IgG1, was immobilized on a Biacore chip. Ang-1-FD-Fc-FD-containing solution was passed over the chip and binding between Tie-2 ectodomain and Ang-1-FD-Fc-FD was allowed to occur. The binding step was followed by a 0.5 M NaCl high salt wash. The high salt wash was not able to disrupt the interaction between the Ang-1-FD-Fc-FD protein and the Tie-2 receptor ectodomain, implying that there is a strong interaction between the mutant ligand and receptor. This result is consistent with earlier Biacore results in which both Ang-1-FD-Fc-FD parent molecule, angiopoietin-1 and the mutant Ang1* molecule, have been shown to interact strongly with the Tie-2-Fc receptor and that this interaction is not disrupted by high salt. In contrast, several mutant molecules derived from the parent angiopoietin-1 molecule are readily dissociated from the Tie-2-Fc receptor when treated with high salt. The mutant molecules, designated Ang-1/FD (a monomer with respect to the FD), Ang-1/FD-Fc (also a monomer with respect to the FD, but which is able to form a dimer due to the presence of the Fc domain), and Ang-1/C/FD (a monomer with respect to the FD, but which also contains the coiled-coil domain of angiopoietin-1), do not exist in multimeric forms sufficient for high affinity binding to the Tie-2 receptor.

<u>Example 3: Characterization of COS cell-derived Ang-2-FD-Fc-FD protein.</u>

<u>Molecular Weight Analysis</u>: As described for Ang-1-FD-Fc-FD *supra*, the predicted molecular weight for Ang-2-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form of Ang-2-FD-Fc-FD has a predicted

weight of 76,052, with three predicted N-linked glycosylation sites that could potentially increase the molecular weight of the monomeric protein to 83,552. Like Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,104. SDS PAGE analyses of COS cell-derived protein confirmed these approximate molecular weights, with a band running at about 200kD under non-reducing conditions and a band running at about 88kD under reducing conditions (Figure 7). Light scatter analysis confirmed the molecular weight (171kD) and revealed that the Ang-2-FD-Fc-FD protein, like Ang-1-FD-Fc-FD, exists as a homogeneous species (Figure 8).

<u>Expression Level in COS Cells:</u> COS cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was generated by transiently transfecting COS cells with the Ang-2-FD-Fc-FD DNA construct described *supra*. The COS cell supernatant was analyzed by Biacore to quantitate the amount of Ang-2-FD-Fc-FD protein present in the supernatant. This analysis resulted in an RU value of 606, which is equivalent to 0.7mg of recombinant protein/liter of COS cell supernatant. These values represent relatively high levels of expression.

<u>Purification of COS Supernatants:</u> As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The COS cell supernatant was purified as described for Ang-1-FD-Fc-FD supra and yielded approximately 2 mg of purified Ang-2-FD-Fc-FD protein that was used in the studies described *infra* to further characterize this protein.

<u>N-terminal sequencing:</u> Purified COS cell-derived Ang-2-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Glu, wherein X is Cys. This

sequence can be found at amino acids 16-20 of Figure 4A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A.

<u>Receptor binding analysis of COS cell-derived protein</u>: To determine whether the COS cell-derived Ang-2-FD-Fc-FD protein could bind to the Tie-2 receptor, standard Biacore analysis was performed as described for Ang-1-FD-Fc-FD *supra*. As with Ang-1-FD-Fc-FD, a high salt wash was not able to disrupt the interaction between the Ang-2-FD-Fc-FD protein and the Tie-2-Fc receptor, again implying that there is a strong interaction between mutant ligand and receptor.

<u>Example 4: Effects of COS cell-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.</u>

Because Ang-1-FD-Fc-FD is a mutant molecule derived from the agonist angiopoietin-1 and Ang-2-FD-Fc-FD is a mutant molecule derived from the antagonist angiopoietin-2, we wanted to determine whether or not these two mutant molecules would retain the activity associated with the parent molecule from which it was derived.

<u>Assay system</u>: All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA <u>80</u>:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill in the art.

<u>(A) Ang1*-mediated vs. Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells</u>: EAhy926 cells were stimulated with either 0.1 μg/ml, 0.2 μg/ml, or 0.8 μg/ml Ang1* or Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 9).

(B) Ability of Ang-2-FD-Fc-FD to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells: EAhy926 cells were treated with 0.4 μg/ml of the Tie-2 agonist Ang1* and 1 μg/ml, 2 μg/ml, 4 μg/ml. 6 μg/ ml, or 8 μg/ml of Ang-2-FD-Fc-FD. As shown in Figure 10, Ang-2-FD-Fc-FD is able to block Ang1* stimulation of the Tie-2 receptor when it is present in at least a 10-15 fold molar excess of Ang1*.

(C) Ability of angiopoietin-2 to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells: To compare the blocking effects of the naturally occurring antagonist angiopoietin-2 with that of Ang-2-FD-Fc-FD, the same experiment described in (B) *supra* was performed, substituting angiopoietin-2 for Ang-2-FD-Fc-FD. The results of this experiment are shown in Figure 11. At a 20 fold molar excess, the angiopoietin-2 has not reduced the phosphorylation level to 50%. This result, coupled with the results described in (B) *supra* implies that Ang-2-FD-Fc-FD is a more potent inhibitor or Ang1*-mediated Tie-2 receptor phosphorylation than angiopoietin-2.

(D) Ability of Ang-2-FD-Fc-FD to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells: EAhy926 cells were treated with 0.2 μg/ml of the naturally occurring Tie-2 agonist angiopoietin-1 and 1 μg/ml, 2 μg/ml, 4 μg/ml. 6 μg/ ml, or 8 μg/ml of Ang-2-FD-Fc-FD. The results of this experiment, shown in Figure 12, show that while there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, Ang-2-FD-Fc-FD seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 10 and described in (B) *supra*.

(E) Ability of angiopoietin-2 to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells: EAhy926 cells were treated with 0.2 μg/ml of the angiopoietin-1 and 1 μg/ml, 2 μg/ml, 4 μg/ml,

6 μg/ ml, or 8 μg/ml of angiopoietin-2. The results of this experiment, shown in Figure 13, show that there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, but, like Ang-2-FD-Fc-FD, angiopoietin-2 seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 11 and described in (C) *supra*.

<u>Example 5: Construction of Ang-1-FD-Fc-FD CHO cell expression vector pRG763/Ang-1-FD-Fc-FD.</u>

The pRG763/Ang-1-FD-Fc-FD CHO cell expression vector was constructed by isolating from the plasmid pCDNA3.1/Ang1-FD-Fc-FD a 2115 base pair EcoRI - NotI fragment containing Ang1-FD-Fc-FD and ligating this fragment into pRG763 vector digested with EcoRI and NotI. A large scale (2L) culture of E. coli DH10B cells carrying the pRG763/Ang-1-FD-Fc-FD plasmid was grown overnight in TB + ampicillin and the plasmid DNA was extracted using a Promega Wizard Plus Maxiprep kit, following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined in a UV spectrophotometer and fluorometer. The plasmid DNA was verified by digestion of aliquots with NcoI and HincII restriction enzymes. All restriction enzyme digest fragments corresponded to the predicted sizes in a 1% agarose gel.

<u>Example 6: Expression of Ang-1-FD-Fc-FD in CHO cells.</u>

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763/Ang-1-FD-Fc-FD using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10%

FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and purified as described *infra*.

Example 7: Construction of Ang-2-FD-Fc-FD CHO cell expression vector pRG763/Ang-2-FD-Fc-FD.

The plasmid pRG763/Ang-2-FD-Fc-FD was constructed by isolating from the plasmid pCDNA3.1/Ang-2-FD-Fc-FD a 2097 base pair EcoRI - NotI fragment containing Ang-2-FD-Fc-FD and ligating this fragment into the pRG763 vector digested with EcoRI and NotI. A large scale (1L) culture of E. coli DH10B cells carrying the pRG763/Ang-2-FD-Fc-FD plasmid was grown overnight in TB + ampicillin and the plasmid DNA was extracted using a Promega Wizard Plus Maxiprep kit, following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined in a UV spectrophotometer and fluorometer. The plasmid DNA was also verified by digestion of plasmid DNA with NcoI and Ppu10I restriction enzymes. All restriction enzyme digest fragments corresponded to the predicted sizes in a 1% agarose gel.

Example 8: Expression of Ang-2-FD-Fc-FD in CHO cells.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763/Ang-2-FD-Fc-FD using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after

adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles purified as described infra.

Example 9: Characterization of stable CHO clone-derived Ang-1-FD-Fc-FD protein.

Molecular Weight Analysis: The predicted molecular weight for stable CHO clone-derived Ang-1-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form (with respect to the Fc) has a predicted weight of 76,349. In addition, there are three predicted N-linked glycosylation sites, approximately 2500 MW/site, that could potentially increase the molecular weight of the monomeric protein to 83,849. Due to the interaction between the Fc components of adjacent molecules, the protein actually exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,698. Subsequent SDS PAGE analyses confirmed these approximate molecular weights, with a band running at about 210kD under non-reducing conditions and a band running at about 85kD under reducing conditions. Light scatter analysis was performed to further confirm the molecular weight and, more importantly, determine whether or not the protein was a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent was monitored with an on line light scattering detector and a refractive index and/or a UV detector. The light scattering detector is a MiniDawn laser light scattering detector was from Wyatt Technology

Corporation (Santa Barbara, CA). This instrument measures static light at three different angles. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) was used to calculate the protein concentration based on either dn/dc (dn = change of refractive index; dc = concentration) or the extinction coefficient of the protein. The molecular weight of protein is then calculated from the angular dependence of light scattering. The results of this analysis show that the dimeric protein appears to be approximately 173.9kD and the presence of a single peak implies that the protein solution is homogenous.

<u>Expression level of Ang-1-FD-Fc-FD in stable CHO clones:</u> CHO cell supernatant containing recombinant Ang-1-FD-Fc-FD protein was generated by stably transfecting CHO cells with the Ang-1-FD-Fc-FD DNA construct described *supra*. The CHO cell supernatant was analyzed by standard ELISA using an anti-human IgG antibody as a capture antibody and an anti-human IgG antibody conjugated to alkaline phosphatase as a reporter antibody to quantitate the amount of Ang-1-FD-Fc-FD protein present in the supernatant. This analysis revealed expression levels of 2-3 pg/cell/day.

<u>Purification of Ang-1-FD-Fc-FD protein derived from stable CHO clone supernatants:</u> Because the Ang-1-FD-Fc-FD protein contains an Fc domain, purification is relatively simple and straight forward using standard Protein A column chromatography (Pharmacia, Inc.) followed by standard size exclusion chromatography (Pharmacia, Inc.). The CHO cell supernatant was purified as described *supra* and the purified ANG-1-FD-Fc-FD protein was used in the studies described *infra* to further characterize the protein.

<u>N-terminal sequencing of stable CHO clone-derived Ang-1-FD-Fc-FD protein:</u> Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal

sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys. This sequence can be found at amino acids 16-20 of Figure 3A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 Figure 3A.

Example 10: Characterization of stable CHO clone-derived Ang-2-FD-Fc-FD protein.

Molecular Weight Analysis: As described for stable CHO clone-derived Ang-1-FD-Fc-FD *supra*, the predicted molecular weight for stable CHO clone-derived Ang-2-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT). The monomeric form of Ang-2-FD-Fc-FD has a predicted weight of 76,052, with three predicted N-linked glycosylation sites that could potentially increase the molecular weight of the monomeric protein to 83,552. Like Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,104. SDS PAGE analyses confirmed these approximate molecular weights, with a band running at about 200kD under non-reducing conditions and a band running at about 85kD under reducing conditions. Light scatter analysis confirmed the molecular weight (176.6kD) and revealed that the stable CHO clone-derived Ang-2-FD-Fc-FD protein, like stable CHO clone-derived Ang-1-FD-Fc-FD, exists as a homogeneous species.

Expression level of Ang-2-FD-Fc-FD derived from stable CHO clones: CHO cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was generated by stably transfecting CHO cells with the Ang-2-FD-Fc-FD DNA construct described *supra*. The CHO cell supernatant was analyzed by standard ELISA using an anti-human IgG antibody as a capture antibody and an anti-human IgG antibody conjugated to alkaline phosphatase as a reporter antibody to quantitate the amount of Ang-2-FD-Fc-FD protein present in the supernatant. This analysis revealed expression levels of approximately 1-2 pg/cell/day.

<u>Purification of stable CHO clone-derived Ang-2-FD-Fc-FD from cell supernatants</u>: As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The CHO cell supernatant was purified as described for stable CHO clone-derived Ang-1-FD-Fc-FD *supra* and was used in the studies described *infra* to further characterize this protein.

<u>N-terminal sequencing of stable CHO clone-derived Ang-2-FD-Fc-FD protein:</u> Purified stable CHO clone-derived Ang-2-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Asp-X-Ala-Glu-Val, wherein X is Cys. This sequence can be found at amino acids 17-21 of Figure 4A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A.

<u>Example 11: Effects of stable CHO clone-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.</u>

<u>Assay system</u>: All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA <u>80</u>:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill in the art.

<u>(A) Ang1*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells</u>: EAhy926 cells were stimulated with 0.4 μg/ml Ang1* or 0.2 μg/ml or 0.4 μg/ml stable CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that or stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 17).

__(B) Ability of stable CHO clone-derived Ang-2-FD-Fc-FD to block stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells:__ EAhy926 cells were treated with 0.2 μg/ml of the Tie-2 agonist Ang-1-FD-Fc-FD and 2 μg/ml, 4 μg/ml, 8 μg/ml or 16 μg/ml of stable CHO clone-derived Ang-2-FD-Fc-FD. As shown in Figure 18, Ang-2-FD-Fc-FD is able to block stable CHO clone-derived Ang-1-FD-Fc-FD stimulation of the Tie-2 receptor when it is present in at least a 40 fold molar excess of stable CHO clone-derived Ang-1-FD-Fc-FD.

__Ephrin ligands:__

In previous experiments (Davis et al., 1994, Science, __266__:816-819; Gale et al., 1996, Neuron __17__:9-19, Gale and Yancopoulos, 1997, Cell Tissue Research __290__:227-241), soluble, unclustered Ephrin-B1-Fc and Ephrin-B2-Fc, which dimerize at their respective Fc domains and therefore are dimeric with respect to either the Ephrin-B1 or Ephrin-B2 ectodomain, failed to induce EphB2 receptor phosphorylation. However, when either molecule was multimerized by pre-clustering with an anti-Fc antibody, they became potent agonists for the EphB2 receptor, as judged by tyrosine phosphorylation of the EphB2 receptor in a COS cell reporter assay. Because multimerization of both Ephrin-B1 and Ephrin-B2 appears to be necessary for induction of receptor phosphorylation, we theorized that a molecule that contained tandem repeats of either Ephrin-B1 or Ephrin-B2 ectodomains fused to an Fc domain, which would be dimeric with respect to the Fc domain but which would be tetrameric with respect to Ephrin ectodomains, might be sufficiently clustered to induce receptor phosphorylation. To test this hypothesis, the following DNA constructs were constructed, recombinant proteins produced, and reporter assays performed.

Construction of tandem Ephrin ectodomain/Fc domain nucleic acid molecules.

All of the following nucleic acid molecules were constructed by standard recombinant DNA techniques (See e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), sequence-verified by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and subcloned into either the mammalian expression pJFE14 (Ephrin-B1-Ephrin-B1-Fc) or pMT21 (Ephrin-B2-Ephrin-B2-Fc), each with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5′ end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

Example 12: Construction of Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules.

(A) Ephrin-B1-Ephrin-B1-Fc: The Ephrin-B1-Ephrin-B1-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B1 (Davis et al., ibid.), which corresponds to nucleotides 1-711 of Figure 14A-Figure 14B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 712-720 of Figure 14B), followed by a second copy of the ectodomain of Ephrin-B1 (corresponding to nucleotides 721-1344 of Figure 14B-Figure 14D), except that in this copy of the Ephrin-B1 ectodomain the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1345-1353 of Figure 14D),

followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1354-2049 of Figure 14D-Figure 14E).

<u>(B) Ephrin-B2-Ephrin-B2-Fc:</u> The Ephrin-B2-Ephrin-B2-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann et al., 1995, Mol. Cell Biol. <u>15</u>:4821-4929), which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E).

As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.

<u>Example 13: Expression of tandem Ephrin recombinant proteins in COS cells.</u>

COS cells were transiently transfected with either the Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules described *supra* using standard transfection techniques known in the art. Two days subsequent to transfection, the growth medium (DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% calf serum) was aspirated and replaced with serum-free medium (DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine). Cell were grown for an additional three days and then the serum-free medium containing the recombinant proteins was collected. Recombinant protein concentration was determined by performing dot blots and comparing the

signal obtained to a standard curve. Once approximate protein concentrations were determined, the Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc recombinant proteins were used in the cell reporter assays described *infra*.

Example 14: Characterization of the COS cell-derived tandem Ephrin ectodomain/Fc domain recombinant proteins.

Reporter Assay: COS cells, which endogenously express the Eph family receptor EphB2 (Gale et al., 1996, Neuron 17:9-19), were used in reporter assays to evaluate the ability of Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc to induce receptor phosphorylation. The assays were performed as previously described (Davis et al., ibid.; Gale et al., ibid.). Briefly, COS cells were grown to 80-90% confluency in standard growth medium described *supra*. After growth, the medium was aspirated, and replaced with serum-free medium (described *supra*) for 1-2 hours prior to treatment with either Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc recombinant protein. The cells were stimulated with 500 ng/ml Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc for 30 minutes at 37°C, with or without affinity purified human IgG1 Fc-specific goat anti-human antibody (Jackson Immunoresearch, West Grove, PA) at a final concentration of 17 µg/ml. This antibody is capable of clustering the Fc tagged fusion. Subsequent to treatment, the COS cells were harvested and cell lysates were prepared as described in Davis, et al. and Gale, et al., *supra*. The EphB2 receptor protein was immunoprecipitated from the cell lysates using an anti-EphB2 antisera (Henkemeyer et al., 1994, Oncogene 9:1001-1014). Immunoprecipitates were resolved by standard SDS PAGE and transferred to PVDF membranes (Millipore) for western blot analysis. The membranes were probed with either anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Institute, Lake Placid, NY) or anti-EphB2 antibodies (Henkemeyer, et al., ibid.) to determine the extent of EphB2

phosphorylation and the relative quantities of EphB2 in the experimental conditions described *supra*.

__Results__: Both Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc were shown to be approximately as active as anti-Fc antibody-clustered Ephrin-B1-Fc in their ability to induce EphB2 receptor phosphorylation in the COS cell reporter assay. Furthermore, if either of the proteins were further clustered with the goat anti-human Fc antibody, they became even more potent in their ability to induce EphB2 receptor phosphorylation. Figure 16 shows the results of this phosphorylation assay.

__Example 15: Construction of Ephrin-B2-Ephrin-B2-Fc CHO expression vector.__

The Ephrin-B2-Ephrin-B2-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann et al., 1995, Mol. Cell Biol. 15:4821-4929), which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E). This molecule was subcloned into the HindIII and NotI polylinker sites in the expression vector pRG763 and was designated pRG763-m(Ephrin-B2)2-Fc. As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.

Example 16: Expression of Ephrin-B2-Ephrin-B2-Fc in CHO-K1 (E1A) cells.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763-m(Ephrin-B2)2-Fc using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and purified as described *supra*.

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of the receptor binding domain of a ligand, the first subunit being fused to the N-terminal end of a multimerizing component, said multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of a ligand.

2. The nucleic acid of claim 1, wherein the receptor binding domains of the first and second subunits are copies of the receptor binding domain of the same ligand.

3. The nucleic acid of claim 1, wherein the receptor binding domains of the first subunit are copies of the receptor binding domain of a different ligand from the receptor binding domains of the second subunit.

4. The nucleic acid of claim 2, wherein the first and second subunits each have one copy of the receptor binding domain of the ligand.

5. The nucleic acid of claim 3, wherein the first and second subunits each have one copy of the receptor binding domain of the ligand.

6. The nucleic acid of claim 2, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

7. The nucleic acid of claim 4, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

8. The nucleic acid of claim 2, wherein the ligand is selected from the group consisting of the EPH family of ligands.

9. The nucleic acid of claim 4, wherein the ligand is selected from the group consisting of the EPH family of ligands.

10. The nucleic acid of claims 1 through 9, wherein the multimerizing component comprises an immunoglobulin derived domain.

11. The nucleic acid molecule of claim 10, wherein the immunoglobulin derived domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

12. A fusion polypeptide encoded by the nucleic acid molecule of claims 1 through 11.

13. A composition comprising a multimer of the fusion polypeptide of claim 12.

14. The composition of claim 13, wherein the multimer is a dimer.

15. A vector which comprises the nucleic acid molecule of claims 1 through 11.

16. An expression vector comprising a nucleic acid molecule of claims 1 through 11, wherein the nucleic acid molecule is operatively linked to an expression control sequence.

17. A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 16, in a suitable host cell.

18. The host-vector system of claim 17, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell or mammalian cell.

19. The host-vector system of claim 17, wherein the suitable host cell is E. coli.

20. The host-vector system of claim 17, wherein the suitable host cell is a COS cell.

21. The host-vector system of claim 17, wherein the suitable host cell is a CHO cell.

22. A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claims 17 through 21, under conditions permitting production of the fusion polypeptide and recovering the polypeptide so produced.

23. A nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises more than one copy of the receptor binding domain of a ligand in tandem, and wherein either the N-terminal or the C-terminal receptor binding domain is also fused to a multimerizing component.

24. The nucleic acid of claim 23, wherein the receptor binding domains are fused contiguously.

25. The nucleic acid of claim 23, wherein the ligand is not a member of the EPH family of ligands.

26. The nucleic acid of claim 24, wherein the ligand is not a member of

the EPH family of ligands.

27. The nucleic acid of claim 23, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

28. The nucleic acid of claim 24, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

29. The nucleic acid of claims 23 through 28, wherein the multimerizing component comprises an immunoglobulin derived domain.

30. The nucleic acid molecule of claim 29, wherein the immunoglobulin derived domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

31. A fusion polypeptide encoded by the nucleic acid molecule of claims 23 through 30.

32. A composition comprising a multimer of the fusion polypeptide of claim 31.

33. The composition of claim 32, wherein the multimer is a dimer.

34. A vector which comprises the nucleic acid molecule of claims 23 through 30.

35. An expression vector comprising a nucleic acid molecule of claims 23 through 30, wherein the nucleic acid molecule is operatively linked to an expression control sequence.

36. A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 35, in a suitable host cell.

37. The host-vector system of claim 36, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell or mammalian cell.

38. The host-vector system of claim 36, wherein the suitable host cell is E. coli.

39. The host-vector system of claim 36, wherein the suitable host cell is a COS cell.

40. The host-vector system of claim 36, wherein the suitable host cell is a CHO cell.

41. A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claims 36 through 40, under conditions permitting production of the fusion polypeptide and recovering the polypeptide so produced.

Fig.1A.

```
          10         20         30         40
           *    *    *    *    *    *    *    *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
___a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

    50         60         70         80         90
     *    *    *    *    *    *    *    *    *
AGA GAC TGT GCA GAT GTA TAT CAA GCT GGT TTT AAT AAA AGT GGA
Arg Asp Cys Ala Asp Val Tyr Gln Ala Gly Phe Asn Lys Ser Gly>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         100        110        120        130
     *    *    *    *    *    *    *    *    *
ATC TAC ACT ATT TAT ATT AAT AAT ATG CCA GAA CCC AAA AAG GTG
Ile Tyr Thr Ile Tyr Ile Asn Asn Met Pro Glu Pro Lys Lys Val>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

   140        150        160        170        180
     *    *    *    *    *    *    *    *    *
TTT TGC AAT ATG GAT GTC AAT GGG GGA GGT TGG ACT GTA ATA CAA
Phe Cys Asn Met Asp Val Asn Gly Gly Gly Trp Thr Val Ile Gln>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         190        200        210        220
     *    *    *    *    *    *    *    *    *
CAT CGT GAA GAT GGA AGT CTA GAT TTC CAA AGA GGC TGG AAG GAA
His Arg Glu Asp Gly Ser Leu Asp Phe Gln Arg Gly Trp Lys Glu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

   230        240        250        260        270
     *    *    *    *    *    *    *    *    *
TAT AAA ATG GGT TTT GGA AAT CCC TCC GGT GAA TAT TGG CTG GGG
Tyr Lys Met Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         280        290        300        310
     *    *    *    *    *    *    *    *    *
AAT GAG TTT ATT TTT GCC ATT ACC AGT CAG AGG CAG TAC ATG CTA
Asn Glu Phe Ile Phe Ala Ile Thr Ser Gln Arg Gln Tyr Met Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

   320        330        340        350        360
     *    *    *    *    *    *    *    *    *
AGA ATT GAG TTA ATG GAC TGG GAA GGG AAC CGA GCC TAT TCA CAG
Arg Ile Glu Leu Met Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

         370        380        390        400
     *    *    *    *    *    *    *    *    *
TAT GAC AGA TTC CAC ATA GGA AAT GAA AAG CAA AAC TAT AGG TTG
Tyr Asp Arg Phe His Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

   410        420        430        440        450
     *    *    *    *    *    *    *    *    *
TAT TTA AAA GGT CAC ACT GGG ACA GCA GGA AAA CAG AGC AGC CTG
Tyr Leu Lys Gly His Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>
```

Fig.1B.

```
       460               470               480               490
        *         *         *         *         *         *         *
ATC TTA CAC GGT GCT GAT TTC AGC ACT AAA GAT GCT GAT AAT GAC
Ile Leu His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    500               510               520               530               540
     *         *         *         *         *         *         *         *
AAC TGT ATG TGC AAA TGT GCC CTC ATG TTA ACA GGA GGA TGG TGG
Asn Cys Met Cys Lys Cys Ala Leu Met Leu Thr Gly Gly Trp Trp>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

             550               560               570               580
     *         *         *         *         *         *         *         *
TTT GAT GCT TGT GGC CCC TCC AAT CTA AAT GGA ATG TTC TAT ACT
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    590               600               610               620               630
     *         *         *         *         *         *         *         *
GCG GGA CAA AAC CAT GGA AAA CTG AAT GGG ATA AAG TGG CAC TAC
Ala Gly Gln Asn His Gly Lys Leu Asn Gly Ile Lys Trp His Tyr>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

             640               650               660               670
     *         *         *         *         *         *         *         *
TTC AAA GGG CCC AGT TAC TCC TTA CGT TCC ACA ACT ATG ATG ATT
Phe Lys Gly Pro Ser Tyr Ser Leu Arg Ser Thr Thr Met Met Ile>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

    680               690               700               710               720
     *         *         *         *         *         *         *         *
CGA CCT TTA GAT TTT GGC CCC GCG CCT TTT AGA GAC TGT GCA GAT
Arg Pro Leu Asp Phe>
____ANG1 FIBRINO_____>
                    Gly Pro Ala Pro>
                    ____GPAP BRI_____>
                                    Phe Arg Asp Cys Ala Asp>
                                    ____ANG1 FIBRINOGEN-_____>

             730               740               750               760
     *         *         *         *         *         *         *         *
GTA TAT CAA GCT GGT TTT AAT AAA AGT GGA ATC TAC ACT ATT TAT
Val Tyr Gln Ala Gly Phe Asn Lys Ser Gly Ile Tyr Thr Ile Tyr>
____d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

    770               780               790               800               810
     *         *         *         *         *         *         *         *
ATT AAT AAT ATG CCA GAA CCC AAA AAG GTG TTT TGC AAT ATG GAT
Ile Asn Asn Met Pro Glu Pro Lys Lys Val Phe Cys Asn Met Asp>
____d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

             820               830               840               850
     *         *         *         *         *         *         *         *
GTC AAT GGG GGA GGT TGG ACT GTA ATA CAA CAT CGT GAA GAT GGA
Val Asn Gly Gly Gly Trp Thr Val Ile Gln His Arg Glu Asp Gly>
____d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>
```

Fig.1C.

```
 860           870           880           890           900
   *     *     *     *     *     *     *     *     *     *
AGT CTA GAT TTC CAA AGA GGC TGG AAG GAA TAT AAA ATG GGT TTT
Ser Leu Asp Phe Gln Arg Gly Trp Lys Glu Tyr Lys Met Gly Phe>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

             910           920           930           940
   *     *     *     *     *     *     *     *     *     *
GGA AAT CCC TCC GGT GAA TAT TGG CTG GGG AAT GAG TTT ATT TTT
Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Ile Phe>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

 950           960           970           980           990
   *     *     *     *     *     *     *     *     *     *
GCC ATT ACC AGT CAG AGG CAG TAC ATG CTA AGA ATT GAG TTA ATG
Ala Ile Thr Ser Gln Arg Gln Tyr Met Leu Arg Ile Glu Leu Met>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

            1000          1010          1020          1030
   *     *     *     *     *     *     *     *     *     *
GAC TGG GAA GGG AAC CGA GCC TAT TCA CAG TAT GAC AGA TTC CAC
Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln Tyr Asp Arg Phe His>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

1040          1050          1060          1070          1080
   *     *     *     *     *     *     *     *     *     *
ATA GGA AAT GAA AAG CAA AAC TAT AGG TTG TAT TTA AAA GGT CAC
Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu Tyr Leu Lys Gly His>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

            1090          1100          1110          1120
   *     *     *     *     *     *     *     *     *     *
ACT GGG ACA GCA GGA AAA CAG AGC AGC CTG ATC TTA CAC GGT GCT
Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu Ile Leu His Gly Ala>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

1130          1140          1150          1160          1170
   *     *     *     *     *     *     *     *     *     *
GAT TTC AGC ACT AAA GAT GCT GAT AAT GAC AAC TGT ATG TGC AAA
Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp Asn Cys Met Cys Lys>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

            1180          1190          1200          1210
   *     *     *     *     *     *     *     *     *     *
TGT GCC CTC ATG TTA ACA GGA GGA TGG TGG TTT GAT GCT TGT GGC
Cys Ala Leu Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

1220          1230          1240          1250          1260
   *     *     *     *     *     *     *     *     *     *
CCC TCC AAT CTA AAT GGA ATG TTC TAT ACT GCG GGA CAA AAC CAT
Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr Ala Gly Gln Asn His>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>
```

Fig.1D.

```
             1270          1280          1290          1300
         *        *     *        *        *      *        *        *      *
GGA AAA CTG AAT GGG ATA AAG TGG CAC TAC TTC AAA GGG CCC AGT
Gly Lys Leu Asn Gly Ile Lys Trp His Tyr Phe Lys Gly Pro Ser>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

  1310          1320          1330          1340          1350
         *        *     *        *        *      *        *        *      *
TAC TCC TTA CGT TCC ACA ACT ATG ATG ATT CGA CCT TTA GAT TTT
Tyr Ser Leu Arg Ser Thr Thr Met Met Ile Arg Pro Leu Asp Phe>
___d___d___d___ANG1 FIBRINOGEN-LIKE DOMAIN_d___d___d___d___>

             1360          1370          1380          1390
         *        *     *        *        *      *        *        *      *
GGA CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA
Gly Pro Gly>
___e___e___>
            Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro>
            ___f___f___f____FC TAG [SPLIT]_f___f___f___f___>

  1400          1410          1420          1430          1440
         *        *     *        *        *      *        *        *      *
CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC
Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

             1450          1460          1470          1480
         *        *     *        *        *      *        *        *      *
TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

  1490          1500          1510          1520          1530
         *        *     *        *        *      *        *        *      *
GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

             1540          1550          1560          1570
         *        *     *        *        *      *        *        *      *
GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC
Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

  1580          1590          1600          1610          1620
         *        *     *        *        *      *        *        *      *
AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>

             1630          1640          1650          1660
         *        *     *        *        *      *        *        *      *
GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG
Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys>
___f___f___f___f___f__FC TAG [SPLIT]___f___f___f___f___f___>
```

Fig.1E.

```
 1670        1680        1690        1700        1710
    *     *     *     *     *     *     *     *     *
GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC
Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

          1720        1730        1740        1750
    *     *     *     *     *     *     *     *     *
GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG
Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

 1760        1770        1780        1790        1800
    *     *     *     *     *     *     *     *     *
GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG
Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

          1810        1820        1830        1840
    *     *     *     *     *     *     *     *     *
GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC
Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

 1850        1860        1870        1880        1890
    *     *     *     *     *     *     *     *     *
GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG
Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

          1900        1910        1920        1930
    *     *     *     *     *     *     *     *     *
ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

 1940        1950        1960        1970        1980
    *     *     *     *     *     *     *     *     *
AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC
Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

          1990        2000        2010        2020
    *     *     *     *     *     *     *     *     *
TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG
Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr>
____f___f____f___f____f__FC TAG [SPLIT]____f___f____f___f____f___>

 2030        2040        2050
    *     *     *     *     *     *
CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
____f___f____f__FC TAG [SPLIT]____f___f____f___>
```

Fig.2A.

```
        10          20          30          40
         *     *     *     *     *     *     *     *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
___a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

   50          60          70          80          90
    *     *     *     *     *     *     *     *     *
AGA GAC TGT GCT GAA GTA TTC AAA TCA GGA CAC ACC ACA AAT GGC
Arg Asp Cys Ala Glu Val Phe Lys Ser Gly His Thr Thr Asn Gly>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         100         110         120         130
    *     *     *     *     *     *     *     *     *
ATC TAC ACG TTA ACA TTC CCT AAT TCT ACA GAA GAG ATC AAG GCC
Ile Tyr Thr Leu Thr Phe Pro Asn Ser Thr Glu Glu Ile Lys Ala>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

  140         150         160         170         180
    *     *     *     *     *     *     *     *     *
TAC TGT GAC ATG GAA GCT GGA GGA GGC GGG TGG ACA ATT ATT CAG
Tyr Cys Asp Met Glu Ala Gly Gly Gly Gly Trp Thr Ile Ile Gln>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         190         200         210         220
    *     *     *     *     *     *     *     *     *
CGA CGT GAG GAT GGC AGC GTT GAT TTT CAG AGG ACT TGG AAA GAA
Arg Arg Glu Asp Gly Ser Val Asp Phe Gln Arg Thr Trp Lys Glu>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

  230         240         250         260         270
    *     *     *     *     *     *     *     *     *
TAT AAA GTG GGA TTT GGT AAC CCT TCA GGA GAA TAT TGG CTG GGA
Tyr Lys Val Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         280         290         300         310
    *     *     *     *     *     *     *     *     *
AAT GAG TTT GTT TCG CAA CTG ACT AAT CAG CAA CGC TAT GTG CTT
Asn Glu Phe Val Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val Leu>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

  320         330         340         350         360
    *     *     *     *     *     *     *     *     *
AAA ATA CAC CTT AAA GAC TGG GAA GGG AAT GAG GCT TAC TCA TTG
Lys Ile His Leu Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         370         380         390         400
    *     *     *     *     *     *     *     *     *
TAT GAA CAT TTC TAT CTC TCA AGT GAA GAA CTC AAT TAT AGG ATT
Tyr Glu His Phe Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

  410         420         430         440         450
    *     *     *     *     *     *     *     *     *
CAC CTT AAA GGA CTT ACA GGG ACA GCC GGC AAA ATA AGC AGC ATC
His Leu Lys Gly Leu Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>
```

Fig.2B.

```
         460         470         480         490
   *      *      *      *      *      *      *      *      *
AGC CAA CCA GGA AAT GAT TTT AGC ACA AAG GAT GGA GAC AAC GAC
Ser Gln Pro Gly Asn Asp Phe Ser Thr Lys Asp Gly Asp Asn Asp>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

  500         510         520         530         540
   *      *      *      *      *      *      *      *      *
AAA TGT ATT TGC AAA TGT TCA CAA ATG CTA ACA GGA GGC TGG TGG
Lys Cys Ile Cys Lys Cys Ser Gln Met Leu Thr Gly Gly Trp Trp>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         550         560         570         580
   *      *      *      *      *      *      *      *      *
TTT GAT GCA TGT GGT CCT TCC AAC TTG AAC GGA ATG TAC TAT CCA
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

  590         600         610         620         630
   *      *      *      *      *      *      *      *      *
CAG AGG CAG AAC ACA AAT AAG TTC AAC GGC ATT AAA TGG TAC TAC
Gln Arg Gln Asn Thr Asn Lys Phe Asn Gly Ile Lys Trp Tyr Tyr>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

         640         650         660         670
   *      *      *      *      *      *      *      *      *
TGG AAA GGC TCA GGC TAT TCG CTC AAG GCC ACA ACC ATG ATG ATC
Trp Lys Gly Ser Gly Tyr Ser Leu Lys Ala Thr Thr Met Met Ile>
___b___b___b__ANG2 FIBRINOGEN-LIKE DOMAIN #1___b___b___b___>

  680         690         700         710         720
   *      *      *      *      *      *      *      *      *
CGA CCA GCA GAT TTC GGG GGC CCC GCG CCT TTC AGA GAC TGT GCT
Arg Pro Ala Asp Phe>
___ANG2 FIBRINO____>
                    Gly Gly Pro Ala Pro>
                    ___GGPAP BRIDGE____>
                                        Phe Arg Asp Cys Ala>
                                        ___ANG2 FIBRINO____>

         730         740         750         760
   *      *      *      *      *      *      *      *      *
GAA GTA TTC AAA TCA GGA CAC ACC ACA AAT GGC ATC TAC ACG TTA
Glu Val Phe Lys Ser Gly His Thr Thr Asn Gly Ile Tyr Thr Leu>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

  770         780         790         800         810
   *      *      *      *      *      *      *      *      *
ACA TTC CCT AAT TCT ACA GAA GAG ATC AAG GCC TAC TGT GAC ATG
Thr Phe Pro Asn Ser Thr Glu Glu Ile Lys Ala Tyr Cys Asp Met>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

         820         830         840         850
   *      *      *      *      *      *      *      *      *
GAA GCT GGA GGA GGC GGG TGG ACA ATT ATT CAG CGA CGT GAG GAT
Glu Ala Gly Gly Gly Gly Trp Thr Ile Ile Gln Arg Arg Glu Asp>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>
```

Fig.2C.

```
  860       870        880        890       900
   *    *    *    *    *    *    *    *    *
GGC AGC GTT GAT TTT CAG AGG ACT TGG AAA GAA TAT AAA GTG GGA
Gly Ser Val Asp Phe Gln Arg Thr Trp Lys Glu Tyr Lys Val Gly>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

          910        920        930        940
   *    *    *    *    *    *    *    *    *
TTT GGT AAC CCT TCA GGA GAA TAT TGG CTG GGA AAT GAG TTT GTT
Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Val>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

  950       960        970        980       990
   *    *    *    *    *    *    *    *    *
TCG CAA CTG ACT AAT CAG CAA CGC TAT GTG CTT AAA ATA CAC CTT
Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val Leu Lys Ile His Leu>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

         1000       1010       1020       1030
   *    *    *    *    *    *    *    *    *
AAA GAC TGG GAA GGG AAT GAG GCT TAC TCA TTG TAT GAA CAT TTC
Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu Tyr Glu His Phe>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

 1040      1050       1060       1070      1080
   *    *    *    *    *    *    *    *    *
TAT CTC TCA AGT GAA GAA CTC AAT TAT AGG ATT CAC CTT AAA GGA
Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile His Leu Lys Gly>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

         1090       1100       1110       1120
   *    *    *    *    *    *    *    *    *
CTT ACA GGG ACA GCC GGC AAA ATA AGC AGC ATC AGC CAA CCA GGA
Leu Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile Ser Gln Pro Gly>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

 1130      1140       1150       1160      1170
   *    *    *    *    *    *    *    *    *
AAT GAT TTT AGC ACA AAG GAT GGA GAC AAC GAC AAA TGT ATT TGC
Asn Asp Phe Ser Thr Lys Asp Gly Asp Asn Asp Lys Cys Ile Cys>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

         1180       1190       1200       1210
   *    *    *    *    *    *    *    *    *
AAA TGT TCA CAA ATG CTA ACA GGA GGC TGG TGG TTT GAT GCA TGT
Lys Cys Ser Gln Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>

 1220      1230       1240       1250      1260
   *    *    *    *    *    *    *    *    *
GGT CCT TCC AAC TTG AAC GGA ATG TAC TAT CCA CAG AGG CAG AAC
Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro Gln Arg Gln Asn>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2_____d___d___d___>
```

Fig.2D.

```
          1270        1280        1290        1300
     *     *     *     *     *     *     *     *     *
ACA AAT AAG TTC AAC GGC ATT AAA TGG TAC TAC TGG AAA GGC TCA
Thr Asn Lys Phe Asn Gly Ile Lys Trp Tyr Tyr Trp Lys Gly Ser>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

  1310        1320        1330        1340        1350
     *     *     *     *     *     *     *     *     *
GGC TAT TCG CTC AAG GCC ACA ACC ATG ATG ATC CGA CCA GCA GAT
Gly Tyr Ser Leu Lys Ala Thr Thr Met Met Ile Arg Pro Ala Asp>
___d___d___d__ANG2 FIBRINOGEN-LIKE DOMAIN#2____d___d___d___>

          1360        1370        1380        1390
     *     *     *     *     *     *     *     *     *
TTC GGA CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC
Phe>
___>
    Gly Pro Gly>
    ___e___e___>
                Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys>
                ____f___f___f___f__FC TAG___f___f___f___f___>

  1400        1410        1420        1430        1440
     *     *     *     *     *     *     *     *     *
CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

          1450        1460        1470        1480
     *     *     *     *     *     *     *     *     *
CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

  1490        1500        1510        1520        1530
     *     *     *     *     *     *     *     *     *
CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT
Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

          1540        1550        1560        1570
     *     *     *     *     *     *     *     *     *
GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

  1580        1590        1600        1610        1620
     *     *     *     *     *     *     *     *     *
GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT
Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

          1630        1640        1650        1660
     *     *     *     *     *     *     *     *     *
GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC
Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>
```

Fig.2E.

```
 1670         1680          1690         1700          1710
    *      *    *      *      *     *      *      *      *
AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC
Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

            1720          1730         1740          1750
    *      *    *      *      *     *      *      *      *
ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

 1760         1770          1780         1790          1800
    *      *    *      *      *     *      *      *      *
CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC
Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

            1810          1820         1830          1840
    *      *    *      *      *     *      *      *      *
CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC
Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

 1850         1860          1870         1880          1890
    *      *    *      *      *     *      *      *      *
ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

            1900          1910         1920          1930
    *      *    *      *      *     *      *      *      *
AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC
Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

 1940         1950          1960         1970          1980
    *      *    *      *      *     *      *      *      *
TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC
Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

            1990          2000         2010          2020
    *      *    *      *      *     *      *      *      *
GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr>
___f___f___f___f___f___f__FC TAG___f___f___f___f___f___f___>

 2030         2040          2050         2060
    *      *    *      *      *     *      *
ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
___f___f___f___f____FC TAG_f___f___f___f___f___>
```

Fig.3A.

```
                10          20          30          40
         *         *    *         *         *    *          *    *    *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
____a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

     50              60          70               80          90
      *        *        *        *         *        *       *       *        *
AGA GAC TGT GCA GAT GTA TAT CAA GCT GGT TTT AAT AAA AGT GGA
Arg Asp Cys Ala Asp Val Tyr Gln Ala Gly Phe Asn Lys Ser Gly>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

               100         110         120         130
      *        *        *        *         *        *       *       *        *
ATC TAC ACT ATT TAT ATT AAT AAT ATG CCA GAA CCC AAA AAG GTG
Ile Tyr Thr Ile Tyr Ile Asn Asn Met Pro Glu Pro Lys Lys Val>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

     140             150             160          170         180
      *        *        *        *         *        *       *       *        *
TTT TGC AAT ATG GAT GTC AAT GGG GGA GGT TGG ACT GTA ATA CAA
Phe Cys Asn Met Asp Val Asn Gly Gly Gly Trp Thr Val Ile Gln>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

               190         200         210         220
      *        *        *        *         *        *       *       *        *
CAT CGT GAA GAT GGA AGT CTA GAT TTC CAA AGA GGC TGG AAG GAA
His Arg Glu Asp Gly Ser Leu Asp Phe Gln Arg Gly Trp Lys Glu>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

     230             240             250          260         270
      *        *        *        *         *        *       *       *        *
TAT AAA ATG GGT TTT GGA AAT CCC TCC GGT GAA TAT TGG CTG GGG
Tyr Lys Met Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

               280         290         300         310
      *        *        *        *         *        *       *       *        *
AAT GAG TTT ATT TTT GCC ATT ACC AGT CAG AGG CAG TAC ATG CTA
Asn Glu Phe Ile Phe Ala Ile Thr Ser Gln Arg Gln Tyr Met Leu>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

     320             330             340          350         360
      *        *        *        *         *        *       *       *        *
AGA ATT GAG TTA ATG GAC TGG GAA GGG AAC CGA GCC TAT TCA CAG
Arg Ile Glu Leu Met Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

               370         380         390         400
      *        *        *        *         *        *       *       *        *
TAT GAC AGA TTC CAC ATA GGA AAT GAA AAG CAA AAC TAT AGG TTG
Tyr Asp Arg Phe His Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu>
____b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>
```

Fig.3B.

```
      410            420            430            440            450
        *       *      *       *       *      *       *       *      *
TAT TTA AAA GGT CAC ACT GGG ACA GCA GGA AAA CAG AGC AGC CTG
Tyr Leu Lys Gly His Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

             460            470            480            490
        *       *      *       *       *      *       *       *      *
ATC TTA CAC GGT GCT GAT TTC AGC ACT AAA GAT GCT GAT AAT GAC
Ile Leu His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

      500            510            520            530            540
        *       *      *       *       *      *       *       *      *
AAC TGT ATG TGC AAA TGT GCC CTC ATG TTA ACA GGA GGA TGG TGG
Asn Cys Met Cys Lys Cys Ala Leu Met Leu Thr Gly Gly Trp Trp>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

             550            560            570            580
        *       *      *       *       *      *       *       *      *
TTT GAT GCT TGT GGC CCC TCC AAT CTA AAT GGA ATG TTC TAT ACT
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

      590            600            610            620            630
        *       *      *       *       *      *       *       *      *
GCG GGA CAA AAC CAT GGA AAA CTG AAT GGG ATA AAG TGG CAC TAC
Ala Gly Gln Asn His Gly Lys Leu Asn Gly Ile Lys Trp His Tyr>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

             640            650            660            670
        *       *      *       *       *      *       *       *      *
TTC AAA GGG CCA AGT TAC TCC TTA CGT TCC ACA ACT ATG ATG ATT
Phe Lys Gly Pro Ser Tyr Ser Leu Arg Ser Thr Thr Met Met Ile>
___b___b___b___ANG1 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

      680            690            700            710            720
        *       *      *       *       *      *       *       *      *
CGA CCT TTA GAT TTT GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA
Arg Pro Leu Asp Phe>
___ANG1 FIBRINO____>
                    Gly Pro Gly>
                    ___c___c___>
                                Glu Pro Lys Ser Cys Asp Lys>
                                ___d___d__FC TAG___d___d___>

             730            740            750            760
        *       *      *       *       *      *       *       *      *
ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA
Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

      770            780            790            800            810
        *       *      *       *       *      *       *       *      *
CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG
Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>
```

Fig.3C.

```
          820             830             840             850
 *         *       *       *       *       *       *       *       *
ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

   860             870             880             890             900
    *       *       *       *       *       *       *       *       *
CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

           910             920             930             940
    *       *       *       *       *       *       *       *       *
GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC
Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

   950             960             970             980             990
    *       *       *       *       *       *       *       *       *
AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC
Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

          1000            1010            1020            1030
    *       *       *       *       *       *       *       *       *
TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1040            1050            1060            1070            1080
    *       *       *       *       *       *       *       *       *
CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG
Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

          1090            1100            1110            1120
    *       *       *       *       *       *       *       *       *
CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG
Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1130            1140            1150            1160            1170
    *       *       *       *       *       *       *       *       *
CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

          1180            1190            1200            1210
    *       *       *       *       *       *       *       *       *
TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG
Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1220            1230            1240            1250            1260
    *       *       *       *       *       *       *       *       *
GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC
Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>
```

Fig.3D.

```
         1270           1280           1290           1300
     *     *     *     *     *     *     *     *     *
TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1310           1320           1330           1340           1350
     *     *     *     *     *     *     *     *     *
CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

         1360           1370           1380           1390
     *     *     *     *     *     *     *     *     *
CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA
His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1400           1410           1420           1430           1440
     *     *     *     *     *     *     *     *     *
GGC GGT GGC GGT TCT GGC GCG CCT TTT AGA GAC TGT GCA GAT GTA
Gly Gly Gly Gly Ser Gly Ala Pro>
___G4S LINKER/ASC BRIDGE (N____>
                                Phe Arg Asp Cys Ala Asp Val>
                                ___ANG1 FIBRINOGEN-LIKE____>

         1450           1460           1470           1480
     *     *     *     *     *     *     *     *     *
TAT CAA GCT GGT TTT AAT AAA AGT GGA ATC TAC ACT ATT TAT ATT
Tyr Gln Ala Gly Phe Asn Lys Ser Gly Ile Tyr Thr Ile Tyr Ile>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

  1490           1500           1510           1520           1530
     *     *     *     *     *     *     *     *     *
AAT AAT ATG CCA GAA CCC AAA AAG GTG TTT TGC AAT ATG GAT GTC
Asn Asn Met Pro Glu Pro Lys Lys Val Phe Cys Asn Met Asp Val>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

         1540           1550           1560           1570
     *     *     *     *     *     *     *     *     *
AAT GGG GGA GGT TGG ACT GTA ATA CAA CAT CGT GAA GAT GGA AGT
Asn Gly Gly Gly Trp Thr Val Ile Gln His Arg Glu Asp Gly Ser>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

  1580           1590           1600           1610           1620
     *     *     *     *     *     *     *     *     *
CTA GAT TTC CAA AGA GGC TGG AAG GAA TAT AAA ATG GGT TTT GGA
Leu Asp Phe Gln Arg Gly Trp Lys Glu Tyr Lys Met Gly Phe Gly>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

         1630           1640           1650           1660
     *     *     *     *     *     *     *     *     *
AAT CCC TCC GGT GAA TAT TGG CTG GGG AAT GAG TTT ATT TTT GCC
Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Ile Phe Ala>
___f___f___f___ANG1 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>
```

Fig.3E.

```
 1670          1680           1690          1700          1710
     *       *      *       *       *      *       *       *      *
ATT ACC AGT CAG AGG CAG TAC ATG CTA AGA ATT GAG TTA ATG GAC
Ile Thr Ser Gln Arg Gln Tyr Met Leu Arg Ile Glu Leu Met Asp>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

          1720          1730          1740          1750
     *       *      *       *       *      *       *       *      *
TGG GAA GGG AAC CGA GCC TAT TCA CAG TAT GAC AGA TTC CAC ATA
Trp Glu Gly Asn Arg Ala Tyr Ser Gln Tyr Asp Arg Phe His Ile>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 1760          1770           1780          1790          1800
     *       *      *       *       *      *       *       *      *
GGA AAT GAA AAG CAA AAC TAT AGG TTG TAT TTA AAA GGT CAC ACT
Gly Asn Glu Lys Gln Asn Tyr Arg Leu Tyr Leu Lys Gly His Thr>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

          1810          1820          1830          1840
     *       *      *       *       *      *       *       *      *
GGG ACA GCA GGA AAA CAG AGC AGC CTG ATC TTA CAC GGT GCT GAT
Gly Thr Ala Gly Lys Gln Ser Ser Leu Ile Leu His Gly Ala Asp>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 1850          1860           1870          1880          1890
     *       *      *       *       *      *       *       *      *
TTC AGC ACT AAA GAT GCT GAT AAT GAC AAC TGT ATG TGC AAA TGT
Phe Ser Thr Lys Asp Ala Asp Asn Asp Asn Cys Met Cys Lys Cys>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

          1900          1910          1920          1930
     *       *      *       *       *      *       *       *      *
GCC CTC ATG TTA ACA GGA GGA TGG TGG TTT GAT GCT TGT GGC CCC
Ala Leu Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly Pro>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 1940          1950           1960          1970          1980
     *       *      *       *       *      *       *       *      *
TCC AAT CTA AAT GGA ATG TTC TAT ACT GCG GGA CAA AAC CAT GGA
Ser Asn Leu Asn Gly Met Phe Tyr Thr Ala Gly Gln Asn His Gly>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

          1990          2000          2010          2020
     *       *      *       *       *      *       *       *      *
AAA CTG AAT GGG ATA AAG TGG CAC TAC TTC AAA GGG CCA AGT TAC
Lys Leu Asn Gly Ile Lys Trp His Tyr Phe Lys Gly Pro Ser Tyr>
____f____f____f____ANG1 FIBRINOGEN-LIKE DOMAIN_f____f____f____f____>

 2030          2040           2050          2060
     *       *      *       *       *      *       *       *
TCC TTA CGT TCC ACA ACT ATG ATG ATT CGA CCT TTA GAT TTT
Ser Leu Arg Ser Thr Thr Met Met Ile Arg Pro Leu Asp Phe>
____f____f____f_ANG1 FIBRINOGEN-LIKE DOMAIN____f____f____f____>
```

Fig.4A.

```
                 10                  20                  30                  40
        *         *         *         *         *         *         *         *         *
ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT
Met Ser Ala Leu Leu Ile Leu Ala Leu Val Gly Ala Ala Val Ala>
___a___a___a___a_TRYPSIN SIGNAL SEQUENCE___a___a___a___a___>

        50                  60                  70                  80                  90
        *         *         *         *         *         *         *         *         *
AGA GAC TGT GCT GAA GTA TTC AAA TCA GGA CAC ACC ACA AAT GGC
Arg Asp Cys Ala Glu Val Phe Lys Ser Gly His Thr Thr Asn Gly>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

                100                 110                 120                 130
        *         *         *         *         *         *         *         *         *
ATC TAC ACG TTA ACA TTC CCT AAT TCT ACA GAA GAG ATC AAG GCC
Ile Tyr Thr Leu Thr Phe Pro Asn Ser Thr Glu Glu Ile Lys Ala>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       140                  150                 160                 170                 180
        *         *         *         *         *         *         *         *         *
TAC TGT GAC ATG GAA GCT GGA GGA GGC GGG TGG ACA ATT ATT CAG
Tyr Cys Asp Met Glu Ala Gly Gly Gly Gly Trp Thr Ile Ile Gln>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

                190                 200                 210                 220
        *         *         *         *         *         *         *         *         *
CGA CGT GAG GAT GGC AGC GTT GAT TTT CAG AGG ACT TGG AAA GAA
Arg Arg Glu Asp Gly Ser Val Asp Phe Gln Arg Thr Trp Lys Glu>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       230                  240                 250                 260                 270
        *         *         *         *         *         *         *         *         *
TAT AAA GTG GGA TTT GGT AAC CCT TCA GGA GAA TAT TGG CTG GGA
Tyr Lys Val Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

                280                 290                 300                 310
        *         *         *         *         *         *         *         *         *
AAT GAG TTT GTT TCG CAA CTG ACT AAT CAG CAA CGC TAT GTG CTT
Asn Glu Phe Val Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val Leu>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

       320                  330                 340                 350                 360
        *         *         *         *         *         *         *         *         *
AAA ATA CAC CTT AAA GAC TGG GAA GGG AAT GAG GCT TAC TCA TTG
Lys Ile His Leu Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

                370                 380                 390                 400
        *         *         *         *         *         *         *         *         *
TAT GAA CAT TTC TAT CTC TCA AGT GAA GAA CTC AAT TAT AGG ATT
Tyr Glu His Phe Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>
```

Fig.4B.

```
 410                 420                 430                 440                 450
  *         *         *         *         *         *         *         *         *
CAC CTT AAA GGA CTT ACA GGG ACA GCC GGC AAA ATA AGC AGC ATC
His Leu Lys Gly Leu Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

          460                 470                 480                 490
  *         *         *         *         *         *         *         *         *
AGC CAA CCA GGA AAT GAT TTT AGC ACA AAG GAT GGA GAC AAC GAC
Ser Gln Pro Gly Asn Asp Phe Ser Thr Lys Asp Gly Asp Asn Asp>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

 500                 510                 520                 530                 540
  *         *         *         *         *         *         *         *         *
AAA TGT ATT TGC AAA TGT TCA CAA ATG CTA ACA GGA GGC TGG TGG
Lys Cys Ile Cys Lys Cys Ser Gln Met Leu Thr Gly Gly Trp Trp>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

          550                 560                 570                 580
  *         *         *         *         *         *         *         *         *
TTT GAT GCA TGT GGT CCT TCC AAC TTG AAC GGA ATG TAC TAT CCA
Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

 590                 600                 610                 620                 630
  *         *         *         *         *         *         *         *         *
CAG AGG CAG AAC ACA AAT AAG TTC AAC GGC ATT AAA TGG TAC TAC
Gln Arg Gln Asn Thr Asn Lys Phe Asn Gly Ile Lys Trp Tyr Tyr>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

          640                 650                 660                 670
  *         *         *         *         *         *         *         *         *
TGG AAA GGC TCA GGC TAT TCG CTC AAG GCC ACA ACC ATG ATG ATC
Trp Lys Gly Ser Gly Tyr Ser Leu Lys Ala Thr Thr Met Met Ile>
___b___b___b___ANG2 FIBRINOGEN-LIKE DOMAIN_b___b___b___b___>

 680                 690                 700                 710                 720
  *         *         *         *         *         *         *         *         *
CGA CCA GCA GAT TTC GGG GGC CCG GGC GAG CCC AAA TCT TGT GAC
Arg Pro Ala Asp Phe>
___ANG2 FIBRINO____>
                    Gly Gly Pro Gly>
                    ___GGPG BRI____>
                                    Glu Pro Lys Ser Cys Asp>
                                    ___d____FC TAG_d___d___>

          730                 740                 750                 760
  *         *         *         *         *         *         *         *         *
AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG
Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

 770                 780                 790                 800                 810
  *         *         *         *         *         *         *         *         *
GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC
Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>
```

Fig.4C.

```
            820        830        840        850
 .     *     *     *     *     *     *     *     *
ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

   860        870        880        890        900
    *     *     *     *     *     *     *     *     *
AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC
Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

         910        920        930        940
    *     *     *     *     *     *     *     *     *
GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC
Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

   950        960        970        980        990
    *     *     *     *     *     *     *     *     *
AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

        1000       1010       1020       1030
    *     *     *     *     *     *     *     *     *
GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA
Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1040       1050       1060       1070       1080
    *     *     *     *     *     *     *     *     *
GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

        1090       1100       1110       1120
    *     *     *     *     *     *     *     *     *
CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1130       1140       1150       1160       1170
    *     *     *     *     *     *     *     *     *
GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC
Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

        1180       1190       1200       1210
    *     *     *     *     *     *     *     *     *
TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG
Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1220       1230       1240       1250       1260
    *     *     *     *     *     *     *     *     *
CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>
```

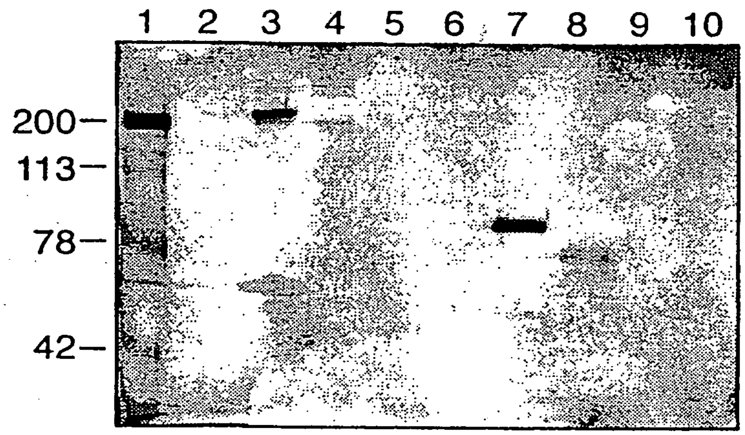
Fig.4D.

```
          1270        1280        1290        1300
         *     *     *     *     *     *     *     *
GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1310        1320        1330        1340        1350
     *     *     *     *     *     *     *     *     *
TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT
Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

          1360        1370        1380        1390
     *     *     *     *     *     *     *     *     *
CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly>
___d___d___d___d___d___d__FC TAG___d___d___d___d___d___d___>

  1400        1410        1420        1430        1440
     *     *     *     *     *     *     *     *     *
AAA GGC GGT GGC GGT TCT GGC GCG CCT AGA GAC TGT GCT GAA GTA
Lys>
___>
    Gly Gly Gly Gly Ser Gly Ala Pro>
    ___e___GGGGSGAP BRIDGE_e___e___>
                                    Arg Asp Cys Ala Glu Val>
                                    ___ANG2 FIBRINOGEN-____>

          1450        1460        1470        1480
     *     *     *     *     *     *     *     *     *
TTC AAA TCA GGA CAC ACC ACA AAT GGC ATC TAC ACG TTA ACA TTC
Phe Lys Ser Gly His Thr Thr Asn Gly Ile Tyr Thr Leu Thr Phe>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

  1490        1500        1510        1520        1530
     *     *     *     *     *     *     *     *     *
CCT AAT TCT ACA GAA GAG ATC AAG GCC TAC TGT GAC ATG GAA GCT
Pro Asn Ser Thr Glu Glu Ile Lys Ala Tyr Cys Asp Met Glu Ala>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

          1540        1550        1560        1570
     *     *     *     *     *     *     *     *     *
GGA GGA GGC GGG TGG ACA ATT ATT CAG CGA CGT GAG GAT GGC AGC
Gly Gly Gly Gly Trp Thr Ile Ile Gln Arg Arg Glu Asp Gly Ser>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

  1580        1590        1600        1610        1620
     *     *     *     *     *     *     *     *     *
GTT GAT TTT CAG AGG ACT TGG AAA GAA TAT AAA GTG GGA TTT GGT
Val Asp Phe Gln Arg Thr Trp Lys Glu Tyr Lys Val Gly Phe Gly>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

          1630        1640        1650        1660
     *     *     *     *     *     *     *     *     *
AAC CCT TCA GGA GAA TAT TGG CTG GGA AAT GAG TTT GTT TCG CAA
Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu Phe Val Ser Gln>
___f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>
```

Fig.4E.

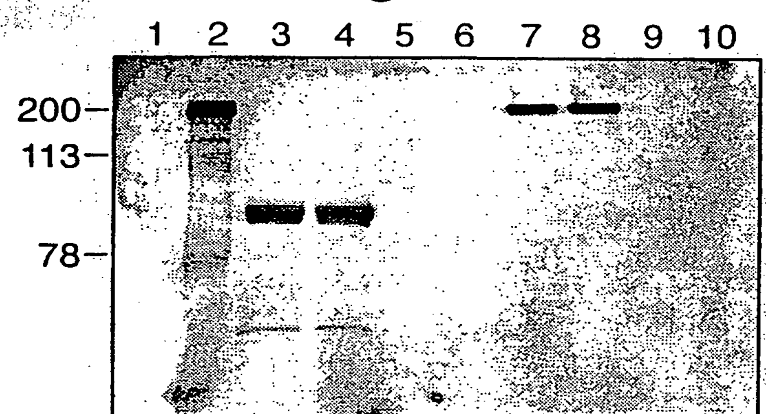
```
   1670           1680            1690           1700          1710
     *       *      *      *       *      *       *      *       *
CTG ACT AAT CAG CAA CGC TAT GTG CTT AAA ATA CAC CTT AAA GAC
Leu Thr Asn Gln Gln Arg Tyr Val Leu Lys Ile His Leu Lys Asp>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

            1720           1730           1740           1750
     *       *      *      *       *      *       *      *       *
TGG GAA GGG AAT GAG GCT TAC TCA TTG TAT GAA CAT TTC TAT CTC
Trp Glu Gly Asn Glu Ala Tyr Ser Leu Tyr Glu His Phe Tyr Leu>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

   1760           1770            1780           1790          1800
     *       *      *      *       *      *       *      *       *
TCA AGT GAA GAA CTC AAT TAT AGG ATT CAC CTT AAA GGA CTT ACA
Ser Ser Glu Glu Leu Asn Tyr Arg Ile His Leu Lys Gly Leu Thr>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

            1810           1820           1830           1840
     *       *      *      *       *      *       *      *       *
GGG ACA GCC GGC AAA ATA AGC AGC ATC AGC CAA CCA GGA AAT GAT
Gly Thr Ala Gly Lys Ile Ser Ser Ile Ser Gln Pro Gly Asn Asp>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

   1850           1860            1870           1880          1890
     *       *      *      *       *      *       *      *       *
TTT AGC ACA AAG GAT GGA GAC AAC GAC AAA TGT ATT TGC AAA TGT
Phe Ser Thr Lys Asp Gly Asp Asn Asp Lys Cys Ile Cys Lys Cys>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

            1900           1910           1920           1930
     *       *      *      *       *      *       *      *       *
TCA CAA ATG CTA ACA GGA GGC TGG TGG TTT GAT GCA TGT GGT CCT
Ser Gln Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly Pro>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

   1940           1950            1960           1970          1980
     *       *      *      *       *      *       *      *       *
TCC AAC TTG AAC GGA ATG TAC TAT CCA CAG AGG CAG AAC ACA AAT
Ser Asn Leu Asn Gly Met Tyr Tyr Pro Gln Arg Gln Asn Thr Asn>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

            1990           2000           2010           2020
     *       *      *      *       *      *       *      *       *
AAG TTC AAC GGC ATT AAA TGG TAC TAC TGG AAA GGC TCA GGC TAT
Lys Phe Asn Gly Ile Lys Trp Tyr Tyr Trp Lys Gly Ser Gly Tyr>
____f___f___f___ANG2 FIBRINOGEN-LIKE DOMAIN_f___f___f___f___>

   2030           2040            2050           2060          2070
     *       *      *      *       *      *       *      *       *
TCG CTC AAG GCC ACA ACC ATG ATG ATC CGA CCA GCA GAT TTC TGA
Ser Leu Lys Ala Thr Thr Met Met Ile Arg Pro Ala Asp Phe>
____f___f___f_ANG2 FIBRINOGEN-LIKE DOMAIN___f___f___f___>
```

Fig.5.



Ang1-FD-Fc-FD

Fig.7.



Ang2-FD-Fc-FD

Fig.6.

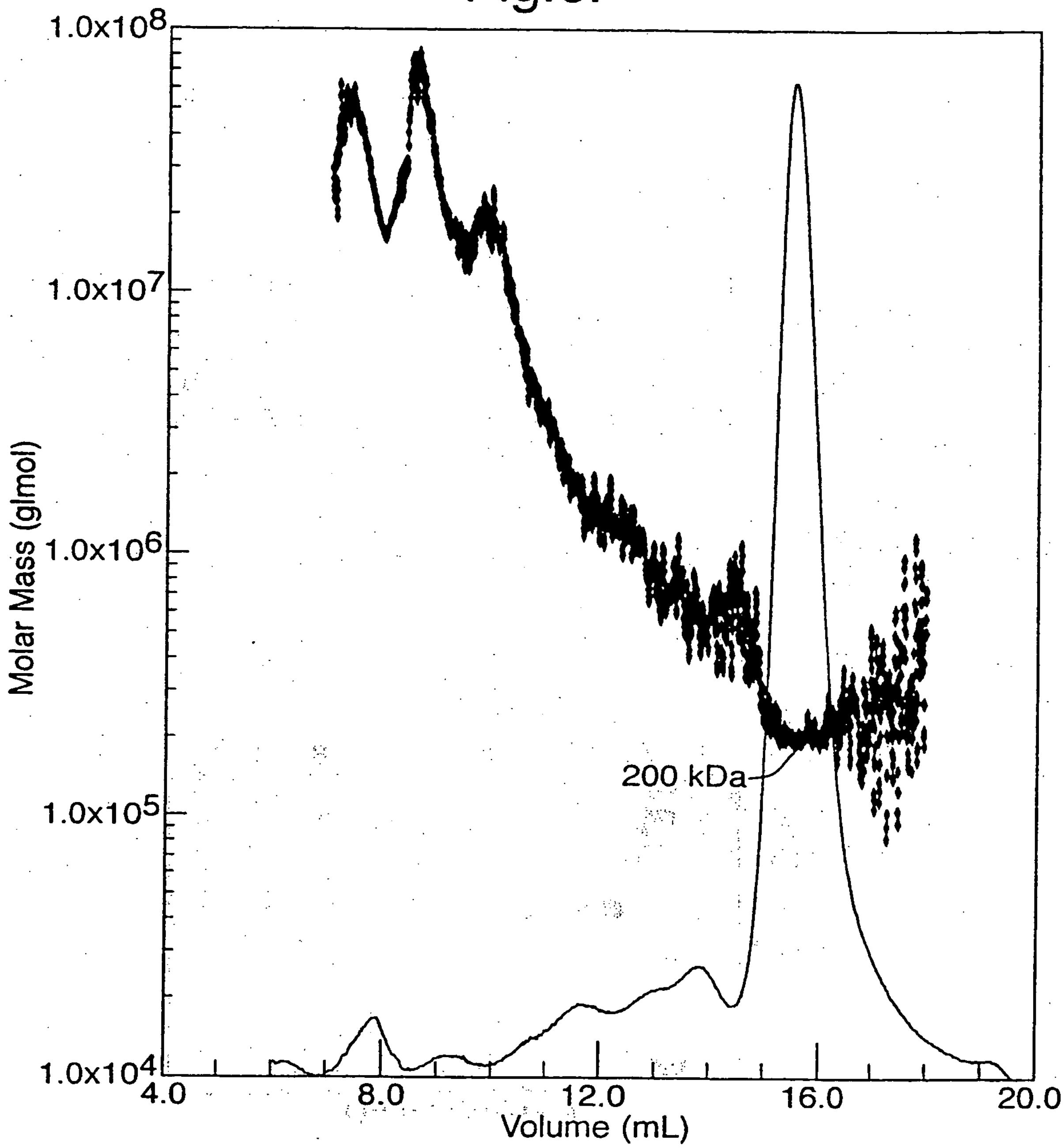

1.0x10^8
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
Molar Mass (glmol)
200 kDa
4.0
8.0
12.0
16.0
20.0
Volume (mL)

Fig.8.

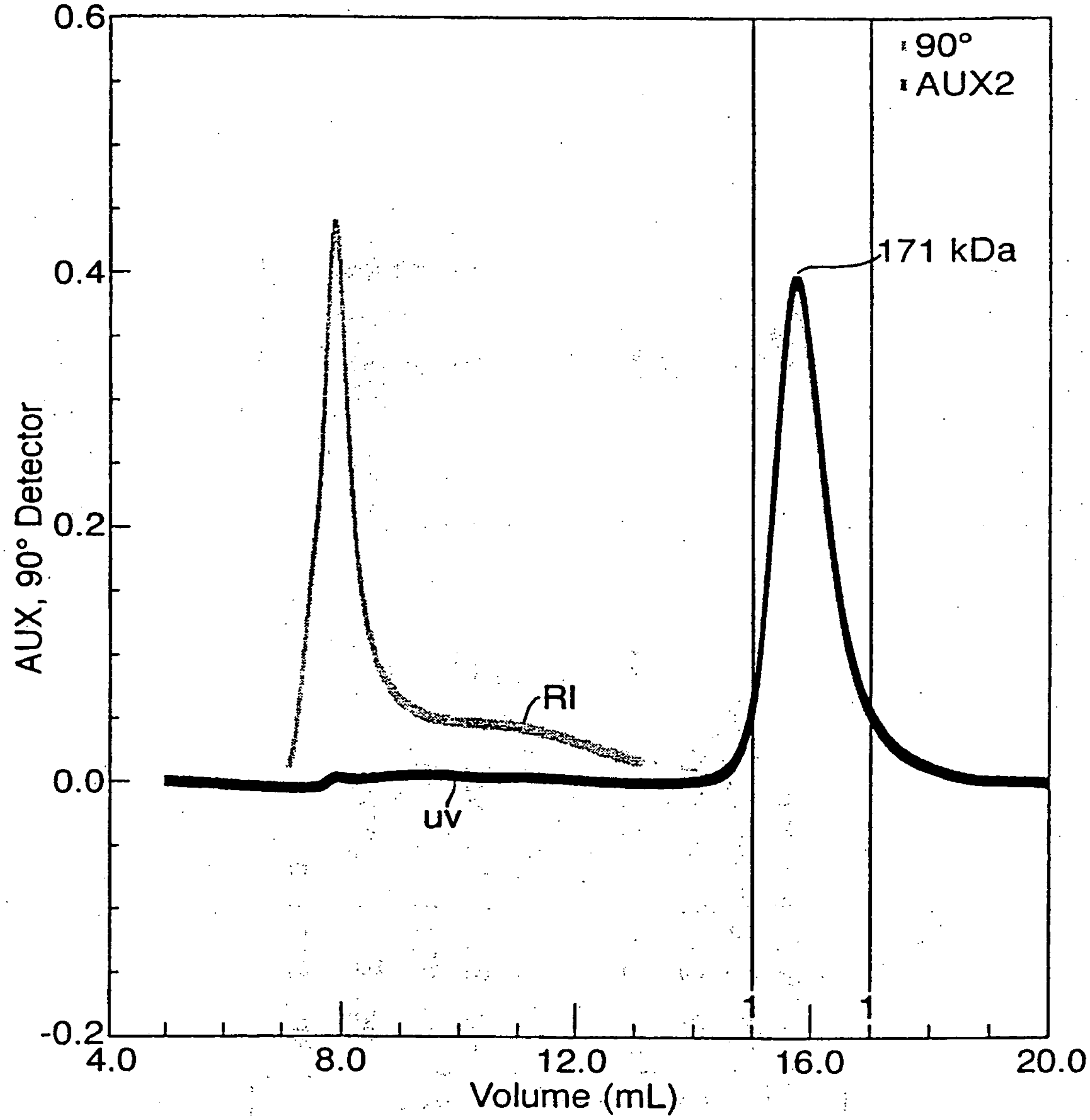

0.6
0.4
0.2
0.0
-0.2
AUX, 90° Detector
90°
AUX2
171 kDa
RI
uv
1
1
4.0
8.0
12.0
16.0
20.0
Volume (mL)

Fig.9.

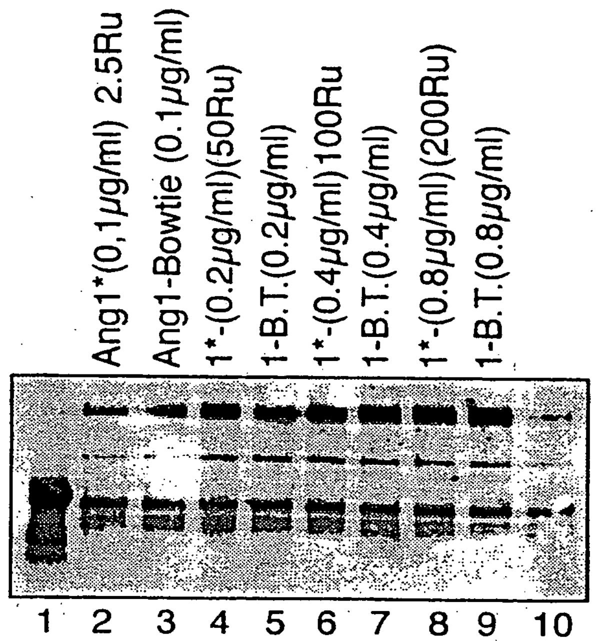

Ang1*(0,1μg/ml) 2.5Ru
Ang1-Bowtie (0.1μg/ml)
1*-(0.2μg/ml)(50Ru)
1-B.T.(0.2μg/ml)
1*-(0.4μg/ml)100Ru
1-B.T.(0.4μg/ml)
1*-(0.8μg/ml)(200Ru)
1-B.T.(0.8μg/ml)
1 2 3 4 5 6 7 8 9 10

Fig.10.

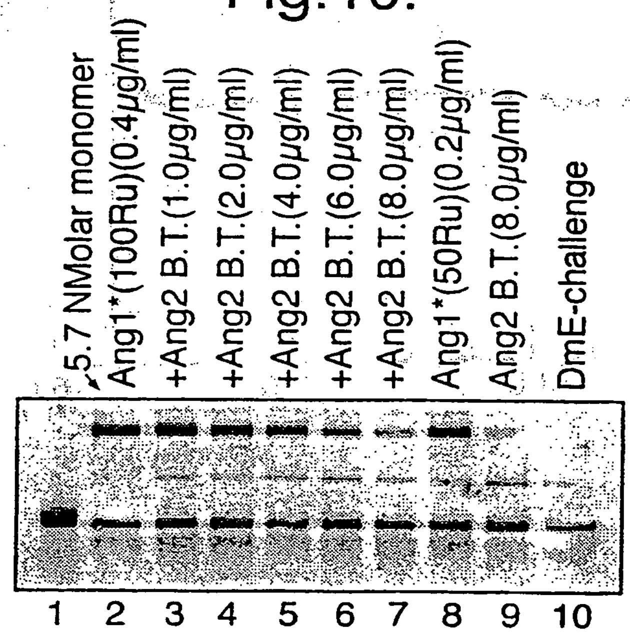

5.7 NMolar monomer
Ang1*(100Ru)(0.4μg/ml)
+Ang2 B.T.(1.0μg/ml)
+Ang2 B.T.(2.0μg/ml)
+Ang2 B.T.(4.0μg/ml)
+Ang2 B.T.(6.0μg/ml)
+Ang2 B.T.(8.0μg/ml)
Ang1*(50Ru)(0.2μg/ml)
Ang2 B.T.(8.0μg/ml)
DmE-challenge
1 2 3 4 5 6 7 8 9 10

Fig. 11.

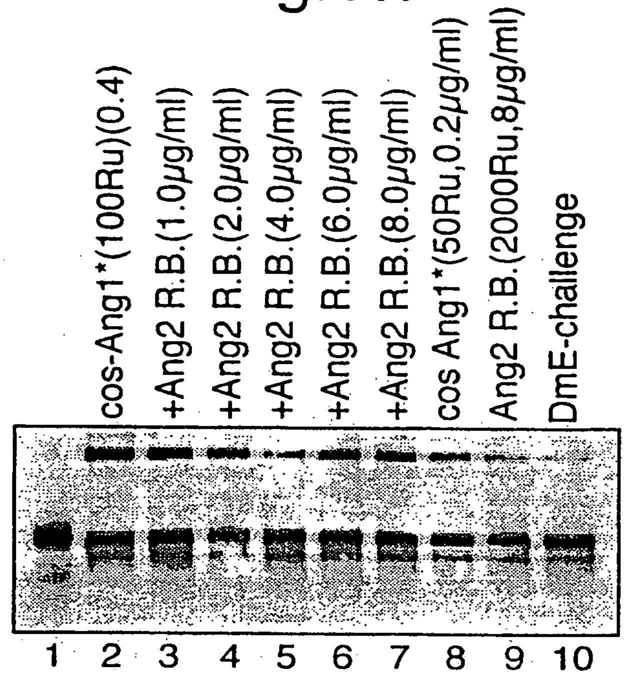

cos-Ang1*(100Ru)(0.4)
+Ang2 R.B.(1.0μg/ml)
+Ang2 R.B.(2.0μg/ml)
+Ang2 R.B.(4.0μg/ml)
+Ang2 R.B.(6.0μg/ml)
+Ang2 R.B.(8.0μg/ml)
cos Ang1*(50Ru,0.2μg/ml)
Ang2 R.B.(2000Ru,8μg/ml)
DmE-challenge
1 2 3 4 5 6 7 8 9 10

Fig. 12.

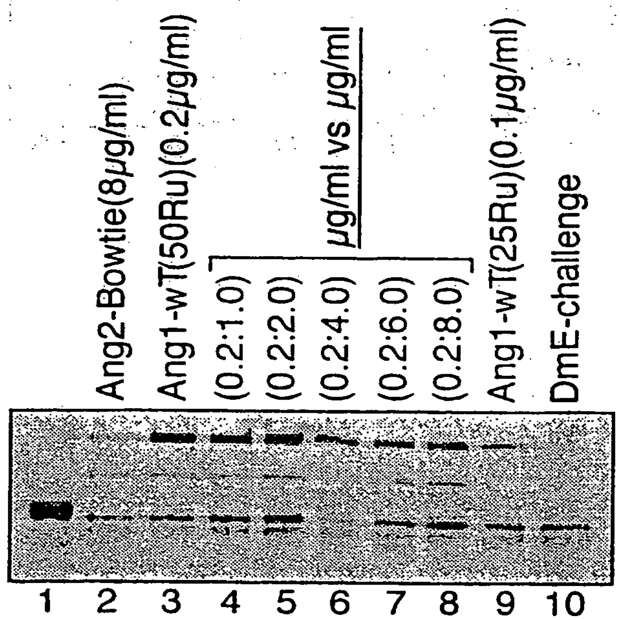

Ang2-Bowtie(8μg/ml)
Ang1-wT(50Ru)(0.2μg/ml)
(0.2:1.0)
(0.2:2.0)
(0.2:4.0)
(0.2:6.0)
(0.2:8.0)
μg/ml vs μg/ml
Ang1-wT(25Ru)(0.1μg/ml)
DmE-challenge
1 2 3 4 5 6 7 8 9 10

Fig.13.

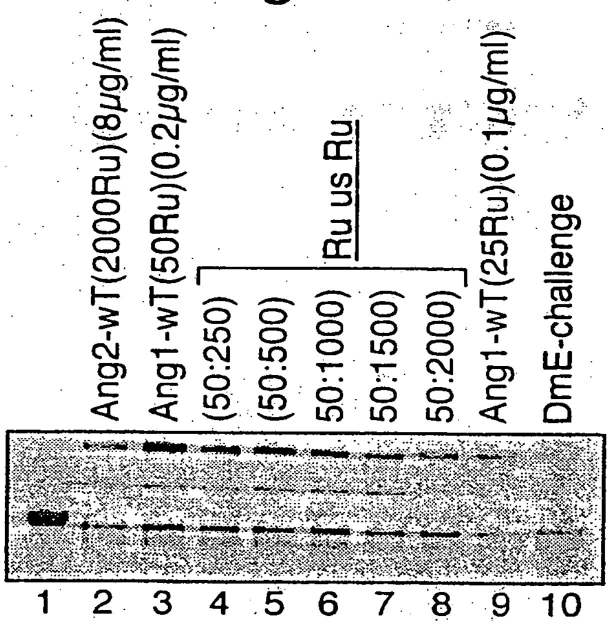

Ang2-wT(2000Ru)(8μg/ml)
Ang1-wT(50Ru)(0.2μg/ml)
(50:250)
(50:500)
50:1000)
50:1500)
50:2000)
Ru us Ru
Ang1-wT(25Ru)(0.1μg/ml)
DmE-challenge
1
2
3
4
5
6
7
8
9
10

Fig.14A.

```
         10         20         30         40
  *      *      *      *      *      *      *      *      *
ATG GCT CGG CCT GGG CAG CGT TGG CTC GGC AAG TGG CTT GTG GCG
Met Ala Arg Pro Gly Gln Arg Trp Leu Gly Lys Trp Leu Val Ala>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

   50         60         70         80         90
  *      *      *      *      *      *      *      *      *
ATG GTC GTG TGG GCG CTG TGC CGG CTC GCC ACA CCG CTG GCC AAG
Met Val Val Trp Ala Leu Cys Arg Leu Ala Thr Pro Leu Ala Lys>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

        100        110        120        130
  *      *      *      *      *      *      *      *      *
AAC CTG GAG CCC GTA TCC TGG AGC TCC CTC AAC CCC AAG TTC CTG
Asn Leu Glu Pro Val Ser Trp Ser Ser Leu Asn Pro Lys Phe Leu>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

  140        150        160        170        180
  *      *      *      *      *      *      *      *      *
AGT GGG AAG GGC TTG GTG ATC TAT CCG AAA ATT GGA GAC AAG CTG
Ser Gly Lys Gly Leu Val Ile Tyr Pro Lys Ile Gly Asp Lys Leu>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

        190        200        210        220
  *      *      *      *      *      *      *      *      *
GAC ATC ATC TGC CCC CGA GCA GAA GCA GGG CGG CCC TAT GAG TAC
Asp Ile Ile Cys Pro Arg Ala Glu Ala Gly Arg Pro Tyr Glu Tyr>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

  230        240        250        260        270
  *      *      *      *      *      *      *      *      *
TAC AAG CTG TAC CTG GTG CGG CCT GAG CAG GCA GCT GCC TGT AGC
Tyr Lys Leu Tyr Leu Val Arg Pro Glu Gln Ala Ala Ala Cys Ser>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

        280        290        300        310
  *      *      *      *      *      *      *      *      *
ACA GTT CTC GAC CCC AAC GTG TTG GTC ACC TGC AAT AGG CCA GAG
Thr Val Leu Asp Pro Asn Val Leu Val Thr Cys Asn Arg Pro Glu>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

  320        330        340        350        360
  *      *      *      *      *      *      *      *      *
CAG GAA ATA CGC TTT ACC ATC AAG TTC CAG GAG TTC AGC CCC AAC
Gln Glu Ile Arg Phe Thr Ile Lys Phe Gln Glu Phe Ser Pro Asn>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

        370        380        390        400
  *      *      *      *      *      *      *      *      *
TAC ATG GGC CTG GAG TTC AAG AAG CAC CAT GAT TAC TAC ATT ACC
Tyr Met Gly Leu Glu Phe Lys Lys His His Asp Tyr Tyr Ile Thr>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>
```

Fig.14B.

```
        410           420            430           440          450
          *      *      *      *      *      *      *      *      *
TCA ACA TCC AAT GGA AGC CTG GAG GGG CTG GAA AAC CGG GAG GGC
Ser Thr Ser Asn Gly Ser Leu Glu Gly Leu Glu Asn Arg Glu Gly>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

                460           470            480           490
          *      *      *      *      *      *      *      *      *
GGT GTG TGC CGC ACA CGC ACC ATG AAG ATC ATC ATG AAG GTT GGG
Gly Val Cys Arg Thr Arg Thr Met Lys Ile Ile Met Lys Val Gly>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

        500           510            520           530          540
          *      *      *      *      *      *      *      *      *
CAA GAT CCC AAT GCT GTG ACG CCT GAG CAG CTG ACT ACC AGC AGG
Gln Asp Pro Asn Ala Val Thr Pro Glu Gln Leu Thr Thr Ser Arg>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

                550           560            570           580
          *      *      *      *      *      *      *      *      *
CCC AGC AAG GAG GCA GAC AAC ACT GTC AAG ATG GCC ACA CAG GCC
Pro Ser Lys Glu Ala Asp Asn Thr Val Lys Met Ala Thr Gln Ala>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

        590           600            610           620          630
          *      *      *      *      *      *      *      *      *
CCT GGT AGT CGG GGC TCC CTG GGT GAC TCT GAT GGC AAG CAT GAG
Pro Gly Ser Arg Gly Ser Leu Gly Asp Ser Asp Gly Lys His Glu>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

                640           650            660           670
          *      *      *      *      *      *      *      *      *
ACT GTG AAC CAG GAA GAG AAG AGT GGC CCA GGT GCA AGT GGG GGC
Thr Val Asn Gln Glu Glu Lys Ser Gly Pro Gly Ala Ser Gly Gly>
____a____a_ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)__a____a____>

        680           690            700           710          720
          *      *      *      *      *      *      *      *      *
AGC AGC GGG GAC CCT GAT GGC TTC TTC AAC TCC AAG GGC CCG GGT
Ser Ser Gly Asp Pro Asp Gly Phe Phe Asn Ser Lys>
____ELK-L ECTODOMAIN 1 (WITH SIGNAL PEPTIDE)_____>
                                                Gly Pro Gly>
                                                ____b____b____>

                730           740            750           760
          *      *      *      *      *      *      *      *      *
AAG AAC CTG GAG CCC GTA TCC TGG AGC TCC CTC AAC CCC AAG TTC
Lys Asn Leu Glu Pro Val Ser Trp Ser Ser Leu Asn Pro Lys Phe>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>

        770           780            790           800          810
          *      *      *      *      *      *      *      *      *
CTG AGT GGG AAG GGC TTG GTG ATC TAT CCG AAA ATT GGA GAC AAG
Leu Ser Gly Lys Gly Leu Val Ile Tyr Pro Lys Ile Gly Asp Lys>
____c____c____c__ELK-L ECTODOMAIN 2 (NO SIGNAL)____c____c____c____>
```

Fig.14C.

```
      820         830         840         850
 .     *     .     *     .     *     .     *     .
CTG GAC ATC ATC TGC CCC CGA GCA GAA GCA GGG CGG CCC TAT GAG
Leu Asp Ile Ile Cys Pro Arg Ala Glu Ala Gly Arg Pro Tyr Glu>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

   860         870         880         890         900
    *     .     *     .     *     .     *     .     *
TAC TAC AAG CTG TAC CTG GTG CGG CCT GAG CAG GCA GCT GCC TGT
Tyr Tyr Lys Leu Tyr Leu Val Arg Pro Glu Gln Ala Ala Ala Cys>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

      910         920         930         940
 .     *     .     *     .     *     .     *     .
AGC ACA GTT CTC GAC CCC AAC GTG TTG GTC ACC TGC AAT AGG CCA
Ser Thr Val Leu Asp Pro Asn Val Leu Val Thr Cys Asn Arg Pro>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

   950         960         970         980         990
    *     .     *     .     *     .     *     .     *
GAG CAG GAA ATA CGC TTT ACC ATC AAG TTC CAG GAG TTC AGC CCC
Glu Gln Glu Ile Arg Phe Thr Ile Lys Phe Gln Glu Phe Ser Pro>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

     1000        1010        1020        1030
 .     *     .     *     .     *     .     *     .
AAC TAC ATG GGC CTG GAG TTC AAG AAG CAC CAT GAT TAC TAC ATT
Asn Tyr Met Gly Leu Glu Phe Lys Lys His His Asp Tyr Tyr Ile>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

  1040        1050        1060        1070        1080
    *     .     *     .     *     .     *     .     *
ACC TCA ACA TCC AAT GGA AGC CTG GAG GGG CTG GAA AAC CGG GAG
Thr Ser Thr Ser Asn Gly Ser Leu Glu Gly Leu Glu Asn Arg Glu>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

     1090        1100        1110        1120
 .     *     .     *     .     *     .     *     .
GGC GGT GTG TGC CGC ACA CGC ACC ATG AAG ATC ATC ATG AAG GTT
Gly Gly Val Cys Arg Thr Arg Thr Met Lys Ile Ile Met Lys Val>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

  1130        1140        1150        1160        1170
    *     .     *     .     *     .     *     .     *
GGG CAA GAT CCC AAT GCT GTG ACG CCT GAG CAG CTG ACT ACC AGC
Gly Gln Asp Pro Asn Ala Val Thr Pro Glu Gln Leu Thr Thr Ser>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

     1180        1190        1200        1210
 .     *     .     *     .     *     .     *     .
AGG CCC AGC AAG GAG GCA GAC AAC ACT GTC AAG ATG GCC ACA CAG
Arg Pro Ser Lys Glu Ala Asp Asn Thr Val Lys Met Ala Thr Gln>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

  1220        1230        1240        1250        1260
    *     .     *     .     *     .     *     .     *
GCC CCT GGT AGT CGG GGC TCC CTG GGT GAC TCT GAT GGC AAG CAT
Ala Pro Gly Ser Arg Gly Ser Leu Gly Asp Ser Asp Gly Lys His>
   c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>
```

Fig.14D.

```
          1270          1280          1290          1300
             *      *      *      *      *      *      *      *
GAG ACT GTG AAC CAG GAA GAG AAG AGT GGC CCA GGT GCA AGT GGG
Glu Thr Val Asn Gln Glu Glu Lys Ser Gly Pro Gly Ala Ser Gly>
___c___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___c___>

   1310          1320          1330          1340          1350
      *      *      *      *      *      *      *      *      *
GGC AGC AGC GGG GAC CCT GAT GGC TTC TTC AAC TCC AAA GGC CCG
Gly Ser Ser Gly Asp Pro Asp Gly Phe Phe Asn Ser Lys>
___c___c__ELK-L ECTODOMAIN 2 (NO SIGNAL)___c___c___>
                                                    Gly Pro>
                                                    ___d___>

          1360          1370          1380          1390
      *      *      *      *      *      *      *      *      *
GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC
Gly>
___>
    Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys>
    ___e___e___e___e__HUMAN IGG1 FC TAG____e___e___e___e___>

   1400          1410          1420          1430          1440
      *      *      *      *      *      *      *      *      *
CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

          1450          1460          1470          1480
      *      *      *      *      *      *      *      *      *
CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

   1490          1500          1510          1520          1530
      *      *      *      *      *      *      *      *      *
ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG
Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

          1540          1550          1560          1570
      *      *      *      *      *      *      *      *      *
TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

   1580          1590          1600          1610          1620
      *      *      *      *      *      *      *      *      *
AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>

          1630          1640          1650          1660
      *      *      *      *      *      *      *      *      *
GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC
Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr>
___e___e___e___e____HUMAN IGG1 FC TAG__e___e___e___e___e___>
```

Fig.14E.

```
 1670          1680           1690           1700           1710
   *       *     *      *       *     *       *      *       *
AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1720           1730           1740           1750
   *       *     *      *       *     *       *      *       *
ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC
Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

 1760          1770           1780           1790           1800
   *       *     *      *       *     *       *      *       *
ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC
Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1810           1820           1830           1840
   *       *     *      *       *     *       *      *       *
CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

 1850          1860           1870           1880           1890
   *       *     *      *       *     *       *      *       *
GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1900           1910           1920           1930
   *       *     *      *       *     *       *      *       *
CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

 1940          1950           1960           1970           1980
   *       *     *      *       *     *       *      *       *
CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

            1990           2000           2010           2020
   *       *     *      *       *     *       *      *       *
TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys>
____e___e___e___e_____HUMAN IGG1 FC TAG__e___e___e___e___e___>

 2030          2040           2050
   *       *     *      *       *
AGC CTC TCC CTG TCT CCG GGT AAA TGA
Ser Leu Ser Leu Ser Pro Gly Lys ***>
____e_____HUMAN IGG1 FC TAG__e___e___>
```

Fig.15A.

```
                10                  20                  30                  40
         *          *        *          *          *        *          *          *          *
ATG GCC ATG GCC CGG TCC AGG AGG GAC TCT GTG TGG AAG TAC TGT
Met Ala Met Ala Arg Ser Arg Arg Asp Ser Val Trp Lys Tyr Cys>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

      50                  60                  70                  80                  90
         *          *        *          *          *        *          *          *          *
TGG GGA CTT TTG ATG GTT TTG TGC AGA ACT GCG ATC TCC AGA TCG
Trp Gly Leu Leu Met Val Leu Cys Arg Thr Ala Ile Ser Arg Ser>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

                100                 110                 120                 130
         *          *        *          *          *        *          *          *          *
ATA GTT TTA GAG CCT ATC TAC TGG AAT TCC TCG AAC TCC AAA TTT
Ile Val Leu Glu Pro Ile Tyr Trp Asn Ser Ser Asn Ser Lys Phe>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

      140                 150                 160                 170                 180
         *          *        *          *          *        *          *          *          *
CTA CCC GGA CAA GGC CTG GTA CTA TAC CCA CAG ATA GGA GAC AAA
Leu Pro Gly Gln Gly Leu Val Leu Tyr Pro Gln Ile Gly Asp Lys>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

                190                 200                 210                 220
         *          *        *          *          *        *          *          *          *
TTG GAT ATT ATT TGC CCC AAA GTG GAC TCT AAA ACT GTT GGC CAG
Leu Asp Ile Ile Cys Pro Lys Val Asp Ser Lys Thr Val Gly Gln>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

      230                 240                 250                 260                 270
         *          *        *          *          *        *          *          *          *
TAT GAA TAT TAT AAA GTT TAT ATG GTT GAT AAA GAC CAA GCA GAC
Tyr Glu Tyr Tyr Lys Val Tyr Met Val Asp Lys Asp Gln Ala Asp>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

                280                 290                 300                 310
         *          *        *          *          *        *          *          *          *
AGA TGC ACA ATT AAG AAG GAG AAT ACC CCG CTG CTC AAC TGT GCC
Arg Cys Thr Ile Lys Lys Glu Asn Thr Pro Leu Leu Asn Cys Ala>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

      320                 330                 340                 350                 360
         *          *        *          *          *        *          *          *          *
AGA CCA GAC CAA GAT GTG AAA TTC ACC ATC AAG TTT CAA GAA TTC
Arg Pro Asp Gln Asp Val Lys Phe Thr Ile Lys Phe Gln Glu Phe>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>

                370                 380                 390                 400
         *          *        *          *          *        *          *          *          *
AGC CCT AAC CTC TGG GGT CTA GAA TTT CAG AAG AAC AAA GAT TAC
Ser Pro Asn Leu Trp Gly Leu Glu Phe Gln Lys Asn Lys Asp Tyr>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)____a____>
```

Fig.15B.

```
   410         420         430         440         450
    *     *     *     *     *     *     *     *     *
TAC ATT ATA TCT ACA TCA AAT GGG TCT TTG GAG GGC CTG GAT AAC

Tyr Ile Ile Ser Thr Ser Asn Gly Ser Leu Glu Gly Leu Asp Asn>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

         460         470         480         490
    *     *     *     *     *     *     *     *     *
CAG GAG GGA GGG GTG TGC CAG ACA AGA GCC ATG AAG ATC CTC ATG
Gln Glu Gly Gly Val Cys Gln Thr Arg Ala Met Lys Ile Leu Met>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

   500         510         520         530         540
    *     *     *     *     *     *     *     *     *
AAA GTT GGA CAA GAT GCA AGT TCT GCT GGA TCA GCC AGG AAT CAC
Lys Val Gly Gln Asp Ala Ser Ser Ala Gly Ser Ala Arg Asn His>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

         550         560         570         580
    *     *     *     *     *     *     *     *     *
GGT CCA ACA AGA CGT CCA GAG CTA GAA GCT GGT ACA AAT GGG AGA
Gly Pro Thr Arg Arg Pro Glu Leu Glu Ala Gly Thr Asn Gly Arg>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

   590         600         610         620         630
    *     *     *     *     *     *     *     *     *
AGT TCA ACA ACA AGT CCC TTT GTG AAG CCA AAT CCA GGT TCT AGC
Ser Ser Thr Thr Ser Pro Phe Val Lys Pro Asn Pro Gly Ser Ser>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

         640         650         660         670
    *     *     *     *     *     *     *     *     *
ACC GAT GGC AAC AGC GCG GGG CAT TCC GGG AAC AAT CTC CTG GGG
Thr Asp Gly Asn Ser Ala Gly His Ser Gly Asn Asn Leu Leu Gly>
____a__EPHRIN-B2  ECTO DOMAIN 1 (WITH SIGNAL PEPTIDE)___a___>

   680         690         700         710         720
    *     *     *     *     *     *     *     *     *
GGC CCG GGA ATA GTT TTA GAG CCT ATC TAC TGG AAT TCC TCG AAC
Gly Pro Gly>
____b___b___>
            Ile Val Leu Glu Pro Ile Tyr Trp Asn Ser Ser Asn>
            ___EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNA_____>

         730         740         750         760
    *     *     *     *     *     *     *     *     *
TCC AAA TTT CTA CCC GGA CAA GGC CTG GTA CTA TAC CCA CAG ATA
Ser Lys Phe Leu Pro Gly Gln Gly Leu Val Leu Tyr Pro Gln Ile>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

   770         780         790         800         810
    *     *     *     *     *     *     *     *     *
GGA GAC AAA TTG GAT ATT ATT TGC CCC AAA GTG GAC TCT AAA ACT
Gly Asp Lys Leu Asp Ile Ile Cys Pro Lys Val Asp Ser Lys Thr>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>
```

Fig.15C.

```
              820         830         840          850
 .     .      .     .      .      .     .      .     .
GTT GGC CAG TAT GAA TAT TAT AAA GTT TAT ATG GTT GAT AAA GAC
Val Gly Gln Tyr Glu Tyr Tyr Lys Val Tyr Met Val Asp Lys Asp>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

  860        870         880        890        900
   .     .     .      .      .     .      .      .
CAA GCA GAC AGA TGC ACA ATT AAG AAG GAG AAT ACC CCG CTG CTC
Gln Ala Asp Arg Cys Thr Ile Lys Lys Glu Asn Thr Pro Leu Leu>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

              910        920         930         940
 .     .      .     .      .      .     .      .     .
AAC TGT GCC AGA CCA GAC CAA GAT GTG AAA TTC ACC ATC AAG TTT
Asn Cys Ala Arg Pro Asp Gln Asp Val Lys Phe Thr Ile Lys Phe>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

  950        960         970        980        990
   .     .     .      .      .     .      .      .
CAA GAA TTC AGC CCT AAC CTC TGG GGT CTA GAA TTT CAG AAG AAC
Gln Glu Phe Ser Pro Asn Leu Trp Gly Leu Glu Phe Gln Lys Asn>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

             1000       1010        1020        1030
 .     .      .     .      .      .     .      .     .
AAA GAT TAC TAC ATT ATA TCT ACA TCA AAT GGG TCT TTG GAG GGC
Lys Asp Tyr Tyr Ile Ile Ser Thr Ser Asn Gly Ser Leu Glu Gly>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

 1040       1050        1060       1070       1080
   .     .     .      .      .     .      .      .
CTG GAT AAC CAG GAG GGA GGG GTG TGC CAG ACA AGA GCC ATG AAG
Leu Asp Asn Gln Glu Gly Gly Val Cys Gln Thr Arg Ala Met Lys>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

             1090       1100        1110        1120
 .     .      .     .      .      .     .      .     .
ATC CTC ATG AAA GTT GGA CAA GAT GCA AGT TCT GCT GGA TCA GCC
Ile Leu Met Lys Val Gly Gln Asp Ala Ser Ser Ala Gly Ser Ala>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

 1130       1140        1150       1160       1170
   .     .     .      .      .     .      .      .
AGG AAT CAC GGT CCA ACA AGA CGC CCA GAG CTA GAA GCT GGT ACA
Arg Asn His Gly Pro Thr Arg Arg Pro Glu Leu Glu Ala Gly Thr>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

             1180       1190        1200        1210
 .     .      .     .      .      .     .      .     .
AAT GGG AGA AGT TCA ACA ACA AGT CCC TTT GTG AAG CCA AAT CCA
Asn Gly Arg Ser Ser Thr Thr Ser Pro Phe Val Lys Pro Asn Pro>
_____EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>

 1220       1230        1240       1250       1260
   .     .     .      .      .     .      .      .
GGT TCT AGC ACC GAT GGC AAC AGC GCG GGG CAT TCC GGG AAC AAT
Gly Ser Ser Thr Asp Gly Asn Ser Ala Gly His Ser Gly Asn Asn>
____ EPHRIN-B2  ECTO DOMAIN 2 ( WITHOUT SIGNAL PEPTIDE)_e___>
```

Fig.15D.

```
          1270           1280        1290        1300
           *        *      *       *       *       *       *       *
CTC CTG GGG G GC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC
                         Glu Pro Lys Ser Cys Asp Lys Thr His>
                         ___c____HUMAN IGG1 FC TAG__c___c___>
          Gly Pro Gly>
         _d__d___d___>
Leu Leu Gly Xxx>
___e___e___e_>

  1310        1320         1330        1340        1350
    *      *      *      *      *      *      *      *      *
ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA
Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser>
___c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1360         1370        1380        1390
    *      *      *      *      *      *      *      *      *
GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser>
___c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

  1400        1410         1420        1430        1440
    *      *      *      *      *      *      *      *      *
CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA
Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu>
___c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1450         1460        1470        1480
    *      *      *      *      *      *      *      *      *
GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG
Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val>
___c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

  1490        1500         1510        1520        1530
    *      *      *      *      *      *      *      *      *
CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG
His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr>
___c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1540         1550        1560        1570
    *      *      *      *      *      *      *      *      *
TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu>
___c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

  1580        1590         1600        1610        1620
    *      *      *      *      *      *      *      *      *
AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA
Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro>
___c___c___c___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>

          1630         1640        1650        1660
    *      *      *      *      *      *      *      *      *
GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA
Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg>
___c___c __c ___c_____HUMAN IGG1 FC TAG__c___c___c___c___c___>
```

Fig.15E.

```
 1670          1680            1690           1700           1710
    *       *       *       *       *       *       *       *       *
GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

         1720          1730           1740           1750
    *       *       *       *       *       *       *       *       *
AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC
Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

 1760          1770            1780           1790           1800
    *       *       *       *       *       *       *       *       *
AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC
Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

         1810          1820           1830           1840
    *       *       *       *       *       *       *       *       *
AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

 1850          1860            1870           1880           1890
    *       *       *       *       *       *       *       *       *
TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG
Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

         1900          1910           1920           1930
    *       *       *       *       *       *       *       *       *
GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC
Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn>
___c___c___c___c____HUMAN IGG1 FC TAG__c___c___c___c___c___>

 1940          1950            1960           1970
    *       *       *       *       *       *       *       *
CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
___c___c___c___c__HUMAN IGG1 FC TAG____c___c___c___c___>
```

Fig.16.

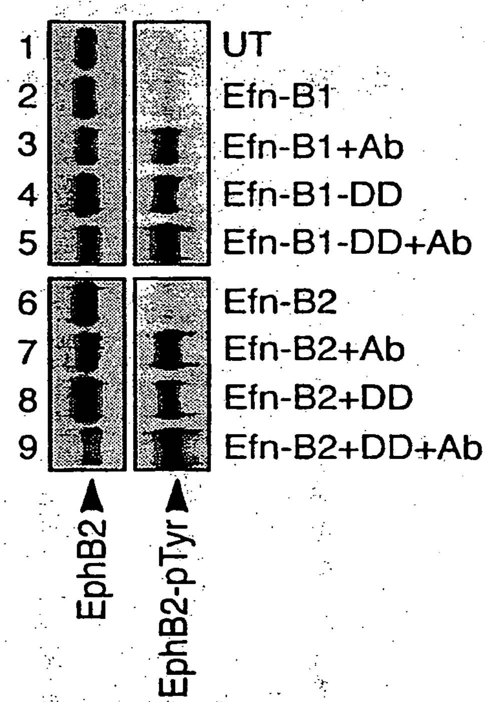

1 UT
2 Efn-B1
3 Efn-B1+Ab
4 Efn-B1-DD
5 Efn-B1-DD+Ab
6 Efn-B2
7 Efn-B2+Ab
8 Efn-B2+DD
9 Efn-B2+DD+Ab
EphB2
EphB2-pTyr

Fig.17.

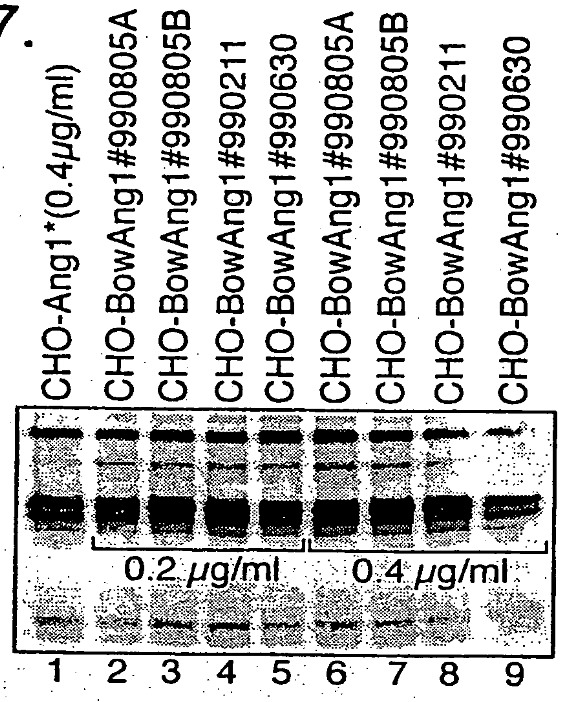
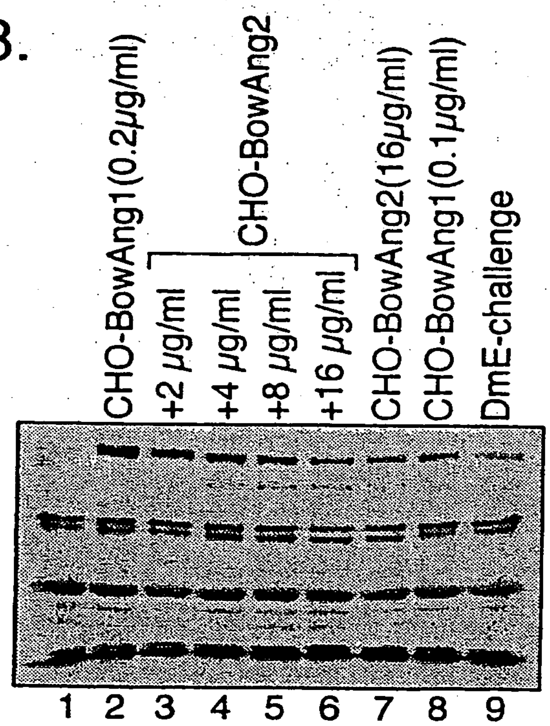

CHO-Ang1*(0.4μg/ml)
CHO-BowAng1#990805A
CHO-BowAng1#990805B
CHO-BowAng1#990211
CHO-BowAng1#990630
CHO-BowAng1#990805A
CHO-BowAng1#990805B
CHO-BowAng1#990211
CHO-BowAng1#990630
0.2 μg/ml
0.4 μg/ml
1 2 3 4 5 6 7 8 9

Fig.18.


CHO-BowAng1(0.2μg/ml)
CHO-BowAng2
+2 μg/ml
+4 μg/ml
+8 μg/ml
+16 μg/ml
CHO-BowAng2(16μg/ml)
CHO-BowAng1(0.1μg/ml)
DmE-challenge
1 2 3 4 5 6 7 8 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/30900

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/62 C12N5/10 C12N1/21 C07K14/515
C07K14/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37621 A (MORPHOSYS PROTEINOPTIMIERUNG ;PACK PETER (DE); HOESS ADOLF (DE)) 28 November 1996 (1996-11-28) abstract page 1, line 12 - line 15 page 2, line 4 - line 9 page 14, line 6 - line 11 page 16, line 29 - line 34 figure 1A	1-5, 12-22
Y		8-11
A		6,7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 May 2000	09/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

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PCT/US 99/30900

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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